Application of proton nuclear magnetic resonance in drug metabolism studies

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APPLICATION OF PROTON NUCLEAR MAGNETIC RESONANCE IN DRUG METABOLISM STUDIES

Thesis

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This research has been carried out in the School of Pharmacy and Pharmacology under the supervision of Dr. L. J. Notarianni and Dr. S.K. Branch.

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TO MY MOTHER AND FATHER

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ABBREVIATIONS

AA	o-Anisic acid
ACE	Acemetacin
ASA	Acetyl salicylic acid, Aspirin
DA	Daunorubicin
DSS	2,2-Dimethyl-2-silapentane-5-sulphonate
EDTA	Ethylenediaminetetraacetic acid
GA	gentisic acid
G6P	Glucose-6-phasphate
G6PDH	Glucose-6-phospate dehydrogenase
HPLC	High performance liquid chromatography
^{1}H NMR	Proton nuclear magnetic resonance
IN	Indomethacin
3 MC	3-Methyl-cholanthrene
MPSH	S-Methyl D-Penicillamine
NADP	Nicotinamide adenine dinucleotide phosphate
P	Paracetamol, Acetaminophen
PB	Phenobarbitone
PC	Paracetamol cysteine
PG	Paracetamol glucuronide
PM	Methoxy paracetamol
PMA	Paracetamol mercapturic acid
PS	Paracetamol sulphate
PSH	D-Penicillamine
PSSC	D-Penicillamine L-cysteine disulfide
PSSP	D-Penicillamine disulfide
SA	Salicylic acid
SAG	Salicyl acyl glucuronide

SPEC	Visible spectrophotometry
SPG	Salicyl phenolic glucuronide
SUA	Salicyluric acid
SUPG	Salicyluric phenolic glucuronide
RA	Rheumathoid arthritis
TCAA	Trichloroacetic acid

SUMMARY

1. Urinary analysis of paracetamol and aspirin from patients taking overdoses have been carried out by high resolution ^{1}H NMR spectroscopy, HPLC and spectrophotometry. The results of the three methods were compared. Quantification of paracetamol by ^{1}H NMR proved to be reliable compared to HPLC. ^{1}H NMR has the advantages of being nondestructive and noninvasive permiting a rapid and simultaneous analysis of concurrently administered drugs, their metabolites and endogenous components which is not the case by other methods. The detection of metabolites in unprocessed urine samples are possible provided they have suitable resonances and are present in at least submillimolar concentrations. In order to concentrate the metabolites for analysis when concentrations are below this, lyophilized urine samples were used.This procedure was used to measure urinary indomethacin, giving good recoveries and reliable results. This method may be applicable for other drugs.

2. D-Penicillamine from pooled EDTA-plasma collected from RA patients has been examined by ^{1}H NMR. Protein binding and the immobility of macromolecules of plasma made quantification of metabolites unreliable. Acid extraction to remove plasma proteins in plasma provided more resolved resonances. However, the low concentration of D-penicillamine and its metabolites in the acid extracted and lyophilized EDTA-plasma, combined with the high 'chemical noise' in the region of interest did not permit quantitative analysis. Despite these problems,

^{1}H NMR analysis of plasma samples may be possible for other drugs present in concentrations greater than 0.1 mM.

3. A study of *in vitro* metabolism in rat liver preparation has been carried out by ^{1}H NMR spectroscopy. The time courses of the metabolism of aminopyrine (DMAP) and daunorubicin (DA) have been monitored by ^{1}H NMR following the disappearance of parent drug and the appearance of its metabolites. The differences between phenobarbitone (PB) and 3-methylcholanthrene (3MC) treated rat livers were distinct in the rate of metabolism observed by NMR of DMAP and DA. This fact allows enzyme induction to be characterized in terms of cytochrome P-450 and cytochrome P-448 using aminopyrine and daunorubicin. This method could be adapted to study *in vitro* metabolism using other inducers or other model drugs, and could be extended for biochemical and toxicological studies.

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Nuclear Magnetic Resonance (NMR) spectroscopy was first reported independently in 1946 by Bloch *et al.*[1] and Purcell *et al.*[2], and the demonstration of this phenomenon as an analytical and research tool has been established since then. Examination of biological material began in the early 1950's with measurement of the water signal in various samples including yeast cells, mammalian blood fractions, fat, liver and muscle tissue[3-6]. In 1959, Singer[7] first demonstrated a method of relating the amplitude of a proton signal to blood flow measurement. This approach is now being coupled with NMR imaging to measure blood flow in large arteries and veins with better accuracy than other methods[8]. Bratton *et al.*(1965)[9] observed a broadening of line width for the water resonance in muscle using pulse NMR techniques to measure T_1 and T_2 relaxation times (described later in section 1.3.3). These initial experiments formed the basis for later development of NMR for biological research and as a diagnostic tool in medicine, such as in the investigations of human cervical mucus, body fluids and tissues[10-12]. Modern high field pulse Fourier Transform NMR instruments have increased sensitivity and the use of specialised pulse sequences has allowed proton NMR studies to be extended from the observation of water to a range of endogenous and exogenous metabolites. Drug metabolism can be followed *in vivo* by proton NMR analysis of urine and other body fluids while cellular suspensions and cell free extracts are used for *in vitro* studies.

Nuclei other than protons have also been studied. Cope (1965)[13] published the first ^{23}Na NMR studies on biological

samples. He suggested that multinuclear studies using ^{1}H, ^{2}D and ^{17}O NMR in the same system would aid in the interpretation of proton NMR data[14]. Since then, many investigators have explored the possibilities of multinuclear NMR experiments on biological samples including ^{1}H, ^{13}C, ^{31}P, ^{19}F, ^{14}N, ^{15}N, ^{23}Na, ^{25}Mg, ^{35}Cl, ^{39}K and ^{43}Ca spectroscopy.

The major role of NMR spectroscopy since its discovery in 1946 is to aid structural elucidation of compounds in synthetic or analytical studies. Even though examination of biological material began in the 1950's, it is only in the 1970's that NMR spectroscopy was extensively developed as a new tool for biological and clinical research. Since then the pace of NMR development has grown rapidly and the advances of high field NMR with increased sensitivity, resolution, and dynamic range has given it possibilities for use in complex drug metabolism studies.

Conventional methods for drug metabolism studies are spectrophotometry and HPLC. The former technique allows only one substance to be assayed at any one time and needs careful sample preparation and can therefore be very time consuming. Although HPLC can be used to assay mixtures, it does not simultaneously provide quantification and absolute identification of the compounds to be separated (reference compounds are needed). NMR has the advantages of being nondestructive and noninvasive and the fact that if the experimental conditions are properly chosen, the various resonances in the spectrum are assignable to chemically different functionalities which serve to aid qualitative and quantitative analysis.

1.2 DRUG METABOLISM

1.2.1 GENERAL

For the majority of drugs, drug metabolism is the single major determinant of its duration of action. Metabolic transformation generally produces more polar compounds than the parent drug, thereby enhancing its excretion from the body. Williams (1971)[15] summarised the possible fate of drugs in the body as follows:

- drugs can undergo enzyme catalysed transformations (e.g. aspirin).
- drugs can be excreted unchanged (e.g. cyclamate).
- drugs can undergo spontaneous reactions when given the appropriate physical conditions, such as pH (e.g. thalidomide).

Most drugs undergo metabolic transformations which are enzyme catalysed in the liver, although other tissues (e.g. lung and kidney) may also participate. Biotransformation or drug metabolism proceeds in two phases, and can be summarised as follows:

Phase I Metabolism

In phase I metabolism, a drug or xenobiotic undergoes reactions such as oxidation, reduction or hydrolysis, yielding more polar products than the parent compound. As a result of these reactions a compound can be made :

- less active or inactive, e.g. oxidation of tolbutamide.
- more active, e.g. prontosil to sulphonilamide.
- converted to a compound which has similar or different activities, e.g. O-dealkylation of codeine to morphine, phenacetin to paracetamol and N-dealkylation of ephedrine to norephedrine.

Introducing chemical groups such as -OH, -COOH or $-NH_2$ to the initial compound allows the next phase of metabolism (phase II metabolism or conjugation) to occur. However, if a drug contains suitable groups, it can undergo phase II metabolism directly. Also, a drug may undergo only phase I metabolism as in the case of ethanol, which is oxidised mainly to CO_2.

Numerous studies have shown that most drugs are oxidised or reduced by enzymes in the endoplasmic reticulum of tissues, mainly those in the liver. The hepatic endoplasmic reticulum is a network of submicroscopic tubules, which extends into almost all regions of the cytoplasm and comprises two major components: a rough-surfaced form consisting of lipoid tubules studded with ribosomes and a smooth-surfaced form lacking in ribosomes[16]. On homogenization, the reticulum disintegrates to form small vesicles which may be isolated as "rough" and "smooth" surfaced "microsomes" by centrifugation using discontinuous gradient systems[17,18]. Smooth-surfaced microsomes metabolize drugs more rapidly than do the rough-surfaced microsomes[17,19].

Microsomal mixed function oxidation reactions are catalysed by non-specific multienzyme systems which have a cytochrome (e.g. cytochrome P-450) as the terminal oxidase. These non-specific cytochromes are located in the endoplasmic reticulum of the cells. For the metabolism of many substrates, the oxidative enzymes require oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as well as reduced cytochrome P-450. Cytochrome P-450, a carbon monoxide-binding pigment of microsomes, is a haemoprotein of the cytochrome "b" type. Unlike most cytochromes it is named, not from the absorption maximum of the reduced form in the visible region, but rather from the unique wavelength of the

absorption maximum of the carbon monoxide (CO) derivative of the reduced form, namely 450 nm. There are at least 11 distinct forms of P-450 which are involved in drug metabolism[20], each variant, although being relatively non-specific, having its own "spectrum" of substrates. In addition, there exsits a second family of cytochrome "P-450" which is distinguished from P-450 by the fact that the reduced cytochrome-CO complex gives a maximum at 448 nm. These cytochromes are consequently named cytochrome P-448 and have different substrate specificities, spectral properties, and electrophoretic protein patterns[21].

Phase II Metabolism

In phase II metabolism, the products of phase I reactions or parent drugs undergo biosynthetic reactions, i.e. conjugations, yielding highly polar or water-soluble compounds and frequently strong organic acids. Conjugation involves the coupling of a compound containing a suitable chemical group (e.g. -OH, $-NH_2$ and -COOH) with an endogenous molecule such as glucuronic acid, glycine, glutathione, methionine and those containing acetyl, sulphate and thio groups.

The conjugation reaction requires an intermediate activated nucleotide, derived from either the conjugation agent or the compound undergoing conjugation, and a transferring enzyme as shown below:

$$\text{Conjugating agent} \xrightarrow{\text{Energy}} \text{Activated conjugating agent nucleotide} \xrightarrow[+\ \text{Transferase}]{\text{Compound}} \text{Conjugated product}$$

$$\text{Compound} \xrightarrow{\text{Energy}} \text{Activated compound nucleotide} \xrightarrow[+\ \text{Transferase}]{\text{Conjugating agent}} \text{Conjugated product}$$

Adenosine triphosphate (ATP) is required as a source of energy to form activated nucleotides. The conjugation enzymes involved in phase II metabolism are located primarily in hepatic microsomes (glucuronic acid), hepatic mitochrondria (glycine), and the soluble fraction of the hepatic cell, kidney and intestinal mucosa (sulphate).

1.2.2 INDUCTION OF DRUG METABOLISING ENZYMES

A number of drugs, insecticides, carcinogens and other chemicals are known to stimulate the activity (synthesis) of drug-metabolizing enzymes in the liver microsomes (e.g. hepatic mixed function oxidase) and this stimulation is referred to as enzyme induction. The injection of small amounts of polycyclic aromatic hydrocarbons, such as 3-methylcholanthrene, 3,4-benzpyrene and 1,2,5,6-dibenzanthracene, rapidly induce several-fold increases in the activities of liver microsomal enzymes that reduce the azo linkage and N-demethylate aminoazo dyes[22]; hydroxylate 3,4-benzpyrene[23] and several drugs[24]; ring-hydroxylate 2-acetylaminofluorene[25] and bind aminoazo dyes in covalent linkage to protein[26]. Evidence was presented in these early studies that polycyclic hydrocarbons increase enzyme activity by inducing the synthesis of more enzyme protein[22,23]. The stimulating effect of barbiturates and other drugs on liver microsomal drug metabolism was discovered independently by Remmer (1958)[27] while investigating mechanisms of barbiturate tolerance, and Conney and Burns (1959)[28] during investigations on the effects of barbiturates and several other unrelated drugs on ascorbic acid biosynthesis and drug metabolism. Chemical compounds which are known to increase the

activity of hepatic mixed function oxidase are of at least two types. The phenobarbitone (PB) type induces the formation of cytochrome P-450 haemoproteins and its associated enzymes, while the 3-methylcholanthrene (3MC) type induces the formation of cytochrome P-448 haemoproteins[29,30]. The relatively less toxic compounds tend to cause an induction pattern similar in nature to that of PB rather than that of 3MC[31]. Thus the characteristic changes in absorbance maxima of the reduced cytochrome-CO complexes in the 448-450 nm region are associated with the pattern of enzyme induction.

The activity of microsomal cytochrome P-450 may be assessed by looking at (a) *in vivo* duration and intensity of drug action when the drug is metabolised primarily by phase I metabolism or (b) measurement of enzyme and drug metabolite concentrations *in vitro*, e.g. N-demethylation of aminopyrene to form first 4-monomethylaminoantipyrene and then 4-aminoantipyrene[32]. Similarly, microsomal cytochrome P-448 activity can be assessed by the above methods, using e.g. daunorubicin which is metabolised by both cytochrome P-450 and P-448.

1.3 METHODS FOR DRUG METABOLISM STUDIES

Numerous methods have been developed to study drug metabolism both qualitatively and quantitatively. Due to the large number of methods which have been used to study drug metabolism, only two methods which are commonly used for the drugs under study will be discussed further, i.e. spectrophotometry (section 1.3.1) and high performance liquid chromatography (section 1.3.2). Spectrophotometry and chromatography are considered traditional methods compared to newer methods such as NMR spectroscopy which

will also be discussed (section 1.3.3).

1.3.1 SPECTROPHOTOMETRY

The simplest assays involve solvent extraction of parent drug and/or metabolic products. Subsequent chemical reaction(s) may be required to develop an ultra-violet absorbance or a coloured or fluorescent derivative of the metabolite. A disadvantage of measuring a compound by spectrophotometry in biological fluids or tissues is the high tissue "blanks" that are often encountered unless the drugs are present in relatively high concentrations.

Colorimetric Analyses

In colorimetric analysis, the procedure involves the measurement of the light absorbance of a compound in the visible region of the spectrum (360 to 900 nm). The majority of drug molecules do not possess an intrinsic absorption in the visible region and it is necessary therefore, to subject such compounds to procedures which result in the formation of a molecular species which has this property. This is generally effected by reacting the drug with another substance to produce a conjugated derivative that is coloured. The type of conjugated derivative formed is dependent upon the functional group(s) of the drug. For example, the chromophore formed by the reaction of nitrous acid with paracetamol was measured quantitatively by its colour in alkaline solution[33]. Paracetamol conjugates, glucuronide and sulphate, have to be converted enzymatically to the parent drug prior to their assay by this method.

In a mixture of drug and its metabolites, the problem of accurate quantitation of each compound becomes even more

complicated and separation is required prior to analysis. However, it is sometimes still possible to assay two closely related compounds individually even if they are not completely separated by extraction procedures. By determining the partition behaviour of the two substances separately under certain fixed conditions, the relative amount of each compound in a mixture can be calculated after determining each concentration under each set of conditions and solving simultaneous equations which define the partition behaviour. This separation technique was applied in the case of salicylic acid, which is soluble in carbon tetrachloride and sparingly soluble in methylene chloride, and salicyluric acid which has the opposite solubility. To determine salicylic acid and some of its metabolites, coloured complex formation, a common method in colorimetric analyses of drugs, was employed, i.e. ferric nitrate ($Fe(NO_3)_3$) was reacted with salicylic acid and salicyluric acid[34] and Folin Ciocalteau's reagent with gentisic acid[35]. Other salicylate metabolites, its phenolic glucuronide and acyl glucuronide, were assayed after converting the metabolites to salicylic acid with enzyme and alkali respectively.

Difference Spectroscopy

Induction of enzyme activity can be assessed by the increased concentration of enzyme protein[22] measured in colorimetric analysis[36]. Another spectrophotometric method used to assess the change of microsomal enzymes (cytochrome P-450/P-448) after induction is difference spectroscopy described below.

In difference spectroscopy, the analyte solution in one state is run against analyte in a different condition and provides a means of cancelling absorptions. Difference spectroscopy has been

used in the characterisation and quantitation of cytochrome P-450/P-448. Cytochrome P-450/P-448, like other haemoproteins, have a characteristic absorption in the visible region. Addition of organic or inorganic ligand results in a perturbation of this spectrum. Since the particulate nature of microsomes gives rise to light scattering, which is a function of wavelength, such changes in the absolute spectrum are seen as fluctuations along a sloping baseline, an undesirable situation for quantitative measurements. In difference spectrosocpy, light scattering, non-specific absorption and the absolute spectrum of the microsomal cytochromes are balanced out by placing microsomes in both cells of a double-beam spectrophotometer, then only the absorption caused by the addition of a ligand to the microsomes in the sample cell is seen as a difference spectrum.

Ultra-Violet (uv) Analyses

For detection of drugs in body fluids, ultra-violet (uv) analysis is applicable to a wide variety of compounds that are capable of absorbing energy from radiations in the wavelength range 200-350 nm. A major problem is the lack of specificity since most compounds absorb in the uv region over a fairly wide range of wavelengths. Nonetheless, the spectrum is usually characteristic for a given compound, named chromophore or chemical derivative, or by the utilization of bathochromic shift to enhance sensitivity and convey specificity. Trevor et al. (1971)[37] claimed that they have usually found uv spectrophotometry to be less sensitive than colorimetry for estimating drugs in body fluids and also encountered more difficulties in eliminating sample blanks.

1.3.2 CHROMATOGRAPHY

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one as a stationary bed of large surface area and the other as a mobile phase that penetrates through or along the stationary bed. Either a liquid or gas may serve as the mobile phase while liquids or solids function as the stationary phase. The mechanism of solute retardation may be adsorption, bonded-phase (reversed phase or polar bonded-phase), ion-exchange or partition. Differences in these properties are exploited to separate components.

Advances in chromatographic instrumentation have brought sophisticated analytical procedures which permit identification by co-elution and subsequent quantification of a drug and/or metabolites after separation from related metabolites and biological constituents. Although many different techniques of chromatography have been used for over 45 years as a separative and analytical technique, high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC) are the most successful combination of chromatographic separation with routine quantitative detection (using standard compounds).

High Peformance Liquid Chromatography (HPLC)

During the last two decades liquid chromatography has been transformed by technological advances, especially in column design and packing, into a remarkably versatile quantitative technique[38,39]. The introduction of chemically bonded phases and reverse phase materials in particular has permitted highly efficient columns for the widespread application of HPLC. In general it has been estimated that 60-80% of all HPLC conditions

involve the use of reverse phase materials[40] which consist of silica covalently bonded with alkyl chains of uniform length. Variations in the composition of the mobile phase produce a range of selectivity to optimise compound separation.

The inherent advantage of HPLC over all other forms of chromatographic techniques lies in the combination of reliability, sensitivity and efficiency coupled with the versatility of retention mechanisms available. The presence of two competing phases (mobile and stationary) for selective interaction with sample molecules allows a wide variation of conditions for separations. In addition, a wide choice of detectors e.g. uv, fluorescence and electrochemical are available for use in HPLC.

In reverse phase HPLC, where the stationary phase is non-polar and the more polar compounds (i.e. metabolites) elute first from the column, advantages lie in its high chemical selectivity and the compatibility with biological materials and aqueous extracts[41]. Minimum sample preparation, such as dilution of the sample followed by selective detection of the compound(s) of interest may be achieved, e.g. in the determination of aspirin and its metabolites[42] and paracetamol[43] in human urine. However, many biological samples especially plasma and hepatocytes require protein removal or compound extraction from biological constituents prior to analysis to prevent deterioration in column performance.

1.3.3 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

1.3.3.1 BASIC PRINCIPLES OF NMR SPECTROSCOPY

The fundamental property of the atomic nucleus involved in NMR is the nuclear spin (I) which has values 0, $^{1}/2$, 1, $1^{1}/2$, etc. in units of $^{h}/2\pi$,(where h is Planck's constant) depending on the mass number and the atomic number of the nucleus. Nuclei for which I = $^{1}/2$ include ^{1}H, ^{19}F, ^{13}C, ^{31}P and ^{15}N. Proton resonance spectra have been studied in the present work and the description in this chapter will be largely confined to them. The nuclear spin interacts with an applied magnetic field such that the nuclear moment can take up only certain allowed orientations. The nuclei of nuclear spin I = $^{1}/2$ can adopt only two orientations in a magnetic field which may be visualised as precession around the axis of the applied magnetic field, B_o, with a frequency ω_o , i.e. the Larmor frequency, and aligned either parallel or antiparallel to it as shown in Figure 1.3.1.

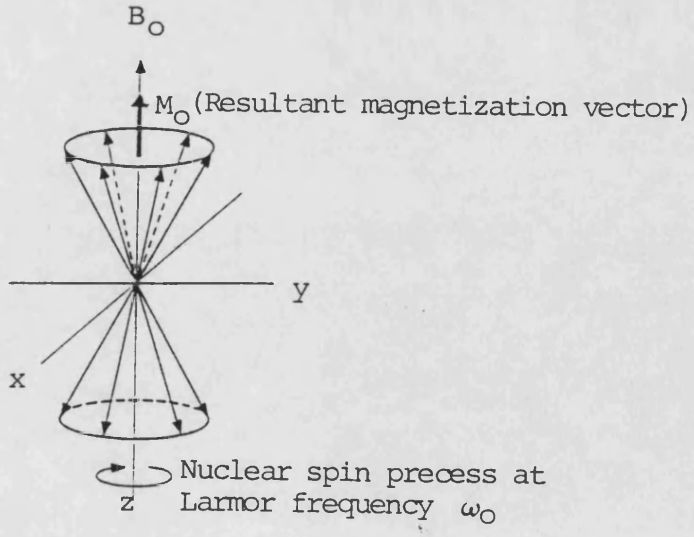


Fig. 1.3.1 Precession of nuclei around an applied magnetic field

Since there is a slight Boltzmann excess of nuclei aligned with the magnetic field, these will give rise to a resultant magnetization vector M_o in the same direction as B_o. The transition energy between these two +z and -z orientations, i.e. the energy required to cause resonance is given by equation 1.3.1.

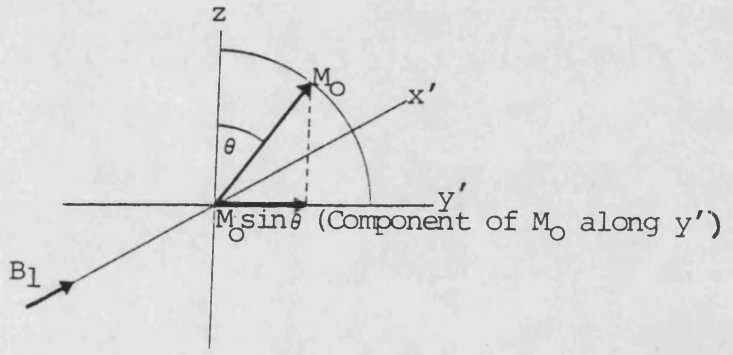
$$\Delta E = \frac{\gamma h B_o}{2\pi} \qquad (1.3.1)$$

where B_o is the external magnetic field strength and γ is magnetogyric ratio, characteristic of a particular nucleus. If B_o is measured in Tesla, the energy, ΔE, has the units Tesla Ampere metre^2 and γ has the units of radian Tesla^{-1} second^{-1}. The intensity of the signal depends on the excess number of spins in the +z direction which in turn is governed by the Boltzmann distribution. From equation 1.3.1 it can be seen that the higher the field strength, the greater ΔE and the higher the excess number of spins along +z thus giving increased sensitivity.

Pulse Fourier Transform (FT) NMR Spectroscopy

To increase the sensitvity of NMR, a great deal of interest has centred on FT NMR spectroscopy in addition to the increasing strength of magnets. In pulse FT NMR the sample is irradiated with a short powerful pulse which contains a range of radiofrequencies (RFs) covering the spectral region of interest. If a pulse of radiofrequency irradiation is applied at the resonant frequency ω_o, along the x axis (see fig. 1.3.1), this would be equivalent to applying a static field (B_1) along x' of the rotating frame. Since the bulk magnetization M_o is stationary in the rotating frame, the effect of applying a constant field along x' would be to cause M_o to rotate in a clockwise direction about the x' with a frequency ω

(Lamor frequency). Hence, if the pulse is applied for t seconds, then M_o will be rotated through an angle θ rad. This is shown in fig. 1.3.2. A spectrometer is normally designed so that it detects signals along the y'-axis as $M_o \sin\theta$.



Field due to the application of RF irradiation at ω_o in laboratory frame

Figure 1.3.2 Effect of applying an RF pulse with Larmor frequency ω_o (the rotating frame reference system) for a time t s on the bulk magnetization vector M_o

All nuclei are excited simultaneously and as they relax back to their equilibrium populations each at different rates, they induce signals of particular frequencies and decay patterns in the receiver coil. The net signal obtained in the time domain, called "free induction decay" (FID), is digitized and stored in a computer. The radiofrequency pulse is repeated and data summated until sufficient have been collected to obtain a spectrum with a satisfactory signal-to-noise ratio. The time domain signal is converted to the frequency domain i.e. the normal spectrum by performing a Fourier transformation on the collected data. The

relaxation occurs by two processes known as spin-lattice (T_1) and spin-spin (T_2) relaxation. The loss of energy from the excited nuclear spins to the surrounding molecular lattice occurs when the vector aligned along the z-axis, M_z, returns to M_o characterized by a relaxation time T_1. In the T_2 process the nuclear spins interchange energy with one another, so that some now precess faster than ω_o while others go slower. The result of this is that the spins begin to fan out in the $x'y'$ plane.

Development of pulse spectrometers was dependent on the availability of computer programs to undertake Fourier transformations on a practical timescale. FT spectrometers allow great flexibility in the type of experiments[44-48], such as spin-echo experiments and two-dimensional NMR that can be performed to obtain extra structural data and information on the dynamics of particular systems.

Spectral Characteristics

The usefulness of NMR spectroscopy for structure elucidation arises because nuclei in different chemical environments resonate at different frequencies according to their local magnetic environment which is related to the electron density around the nucleus. The s-electrons in a molecule produce a magnetic field at the nucleus which is smaller and opposite to the external field. The difference between these fields, called nuclear shielding, produces an upfield shift of the resonance called a diamagnetic shift. Electrons in p-orbitals and other non-spherical orbitals produce larger magnetic fields at the nucleus which give a deshielding effect or low-field shift called the paramagnetic shift.

Resonance frequencies are always measured from a suitable reference compound, normally TMS (Tetramethylsilane) in organic solvents or DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate or TSP) in water. The parameter used to indicate the position of a signal in a spectrum relative to the reference signal is chemical shift, δ, and is defined by equation 1.3.2.

$$\delta = \frac{(\nu \text{ sample} - \nu \text{ reference})}{\text{radio frequency applied}} \times 10^6 \text{ ppm} \qquad (1.3.2)$$

Basic frequency data (ν) of a spectrum must be quoted with the operating frequency of the spectrometer but chemical shift data are independent of the frequency. NMR spectra can also yield information on the connectivity of functional groups in the molecule because signals from one nucleus may be split into particular patterns depending on the number of neighbouring spins. The coupling constants (or J values) can be measured from the splittings if the spectrum is first order i.e. where the couplings are much less than the chemical shift separations.

1.3.3.2 NMR TECHNIQUES FOR DRUG METABOLISM STUDIES

Quantitative Analysis

Proton NMR can be used quantitatively by electronic integration of peaks as the area under a signal is proportional to the number of nuclei in the sample causing that signal, or by peak heights if the line widths are identical. Internal or external standards can be employed. Quantitative analysis is often hampered by the lack of sensitivity due to the small excess number of spins in the +z direction available to absorb radiation energy. Pulse FT NMR spectrometers and higher magnetic fields now offer greater

sensitivity and thus have been applied to the quantitative analysis of biological fluids. In addition, high field instruments have greater resolving power required for complex mixtures.

Pulse FT NMR has advantages for quantitative measurements since pulse repetition can increase the ratio of signal to noise if the pulse delay is sufficient to allow full relaxation. When a pulse of radiofrequency is applied to a spin system, the magnetization along the z-axis decreases (see section 1.3.3.1). If a second pulse is applied immediately, there is smaller M_z magnetization to tip into the y-direction, and consequently a smaller signal is acquired as a result. It is therefore important to apply a sufficiently long pulse interval, which is dependent on the relaxation time, to allow efficient detection of the NMR signal. The unpaired electrons of paramagnetic molecules or ions, and immobile molecules have a very strong effect on relaxation and cause broad and poorly resolved peaks unless precautions are taken to exclude them.

Water Suppression

A problem which arises from the applications of FT NMR technique on the direct analysis of molecules in dilute aqueous solution such as biological fluids is the large water resonance. It makes it difficult to detect much weaker resonances from the solute molecules because of the limited instrumental dynamic range. Several methods have been used to overcome this problem by eliminating the water resonance and will be further discussed:

a) **Fast Pulsing**[49-51]

If the sample has a short relaxation time relative to water, which is usual in proton spectroscopy of biomolecules, the nuclei may be pulsed at a rate which does not allow the water to relax completely while still allowing the sample nuclei to relax. The water resonance may be reduced to one-quarter of its normal height by this means.

b) **Selective Saturation**[52-60]

Secondary irradiation is applied with sufficient power to saturate selectively the water resonance between the end of accumulation of one FID and the next pulse. This power is gated with the irradiation switched off during acquisition to minimise radiofrequency interference (homogated decoupling). The analytical pulse is then applied before the water recovers equilibrium and consequently is much reduced.

c) **WEFT (Water-Eliminated Fourier Transform)**[61-70]

This method capitalizes on the large difference in spin-lattice relaxation times (T_1) exhibited by the resonances of biological macromolecues (<1.0 sec) and that of HDO (5-15 sec), which arises from exchange of protons of the sample or H_2O with D_2O. An inversion recovery pulse sequence of the form T- 180° (x)-τ-90°(x)-acquisition, eliminates HDO from the spectrum. T is a relatively long initial waiting time, followed by a 180° pulse and τ is the time required for the HDO resonance to attain zero net longitudinal magnetization. The 90° pulse results in an FID coming only from the sample protons. It is then theoretically necessary to wait for a

long time ($5T_1$) for the whole system to equilibrate before another pulse sequence can be applied. In practice, however, it is often possible to repeat the process after a shorter time in order to achieve maximum sensitivity in a given time.

d) **Selective excitation**[67,71-78]

Selective pulse sequences are applied to excite only the desired spectral regions, leaving the solvent magnetization oriented along the static field direction, z. Selective excitation methods consist of either long, weak ('soft') pulses[71-74] or short, strong ('hard') pulses separated by delays[67,75-78].

e) **WATR (Water Attenuation by T_2 relaxation)**[79-82]

The spin-spin relaxation time (T_2) of the water protons is selectively reduced by chemical exchange, and the ^1H NMR spectrum is measured by the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence $90^\circ(x)-(\tau-180^\circ(y)-\tau)_n$-acquisition[129], where 90° and 180° are the pulses and τ is the interval time between the pulses. By making the time between the 90° pulse and acquisition of the FID long enough, the water resonance is completely eliminated. Compounds which have labile protons can be used as reagents to selectively decrease the T_2 of the water protons by chemical exchange such as ammonium chloride, guanidium chloride, urea and hydroxylamine hydrochloride whose rate of exchanges are dependent on pH and concentration[82].

Of the various techniques described above, those employing selective excitation are probably the most successful[78] in suppressing the water signal, but they have disadvantages in that resonances near that of water cannot be observed and they require careful pulse adjustment. In the WEFT technique, nuclei whose resonances are in this region can be observed if their T_1 relaxation times are different from those of water protons. However, it should be performed with caution in H_2O since $T_1(H_2O)$ is considerably less than T_1 (HDO), and field homogeneity and accuracy of pulse length are also far more critical, thus the conditions for complete recovery of the sample magnetization become more stringent. The selective saturation stands out as being simple and easy to use and does not need addition of chemicals as in the WATR technique, unless there are no significant resonances near the water resonance since they might be slightly irradiated.

Resolution Enhancement

Proton NMR suffers from a rather narrow chemical shift range which means that there can be problems caused by overlap of resonances from different molecules present in the same sample. This problem can be minimised by using spectrometers with higher magnetic fields (e.g. 400 MHz) and a technique known as "resolution enhancement". The common approach to resolution enhancement is to multiply the FID by a function that de-emphasizes its beginning and emphasizes, at least relatively, its tail before doing the Fourier transformation[83]. Various functions have been developed for this purpose e.g. trapezoidal window,[83] exponential window[84], convolution difference[85], sine-bell routine[86], and Gaussian function[48] as used in present work. The enhancement is paid for by reduced

signal-to-noise ratios and distortions of the spectra which vary in extent with the method.

Spin Echo Experiments

The spin echo experiment is of central importance to modern pulse NMR. The Hahn spin echo method[87] was first used for the measurement of "natural" T_2 values (see section 1.1) in liquids. In this method, a 90°-τ-180° -τ pulse sequence is used (cf. section 1.3.3.2.e). At time 2τ, a spin-echo signal is observed. The Hahn method does not yield true T_2's if there is molecular diffusion. Hence Carr and Purcell (CP)[88] modified this method using the pulse sequence $90^{\circ}(x)$-$(\tau - 180^{\circ}(x) - \tau)_n$- acquisition. All the radiofrequency (RF) pulses are applied along the x-axis and the echo signals will be at 2τ, 4τ, 6τ etc. A further modification was described by Meiboom and Gill[80] using identical conditions to the CP pulse sequence except that a phase shift of 90° was introduced in all the 180° pulses. The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (see section 1.3.3.2.e) gives the same spin-echo signals, even in phase-sensitive detection mode, since it compensates the slight mis-settings of the 180° pulse lengths.

In the basic spin echo method (see fig. 1.3.3), a 90° pulse is initially applied to the spin system. After a time τ, the individual magnetic vectors "fan-out" and lose "phase coherence", both due to spin-spin relaxation (T_2) and magnetic field inhomogeneity. These magnetic vectors will be inverted if a second 180° pulse is applied. Since the rotational sense of their motion is unchanged, the vectors refocus along the y'-axis and a spin echo signal is recorded after the time 2τ. The amplitude of the signal now differs from the initial value only through true

relaxation losses.

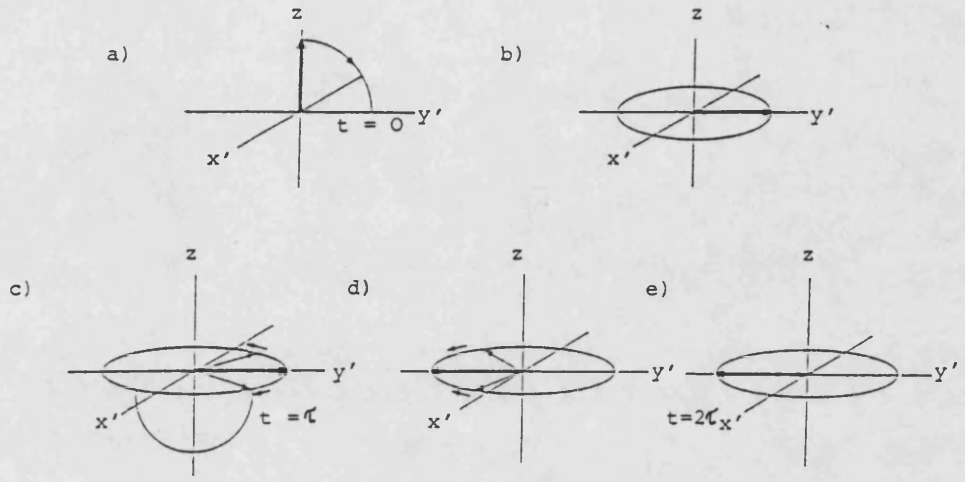


Fig. 1.3.3 The spin echo experiment: a) application of a 90°(x) pulse; b) tansverse magnetization after 90°(x) exitation pulse; c) fanning-out of the magnetization vectors after time τ; d) application of a 180° pulse; e) refocused vector after time 2τ.

It follows that the spin echo experiment causes cancellation of all effects that result from different Larmor frequencies leading to a fanning-out of the macroscopic transverse magnetization (T_2 relaxation) after the exiting pulse. This cancellation can be used to eliminate broad signals since they have short T_2 values[89,90].

A situation distinct from that described above is met if the spin-spin coupling (J) to a neighbouring nucleus occurs leading to the modulation of the signal. At a τ value of 60 ms ($^1/2$ J) doublets with J approximately equal to 8 Hz are inverted, whereas singlets and triplets are upright[91]. This makes it a useful assignment aid. However, the phase modulation of coupled signals in

spin echo experiment causes the distortion of the signals, thus quantitative measurement can be difficult.

1.3.4 OTHER ANALYTICAL METHODS

In addition to the three methods discussed above, i.e. uv-vis spectrophotometry, HPLC and NMR spectroscopy, other methods such as fluorescence spectrophotometry, gas liquid chromatography (GLC) and radioimmunoassay (RIA) may be used in drug metabolism studies. The analytical potency of fluorescence technique is based on the emission of light quanta (fluorescence) by a drug following its absorption of electromagnetic radiation in the uv-vis region. In gas liquid chromatography, the drugs or metabolites to be determined are carried at different rates by an inert gas through a column packed with a support material coated with a non-volatile liquid as the stationary phase. Radioimmunoassay is based on the competition between drug and radiolabelled drug binding to antibody. Thus the proportion of radiolabelled drug bound to the antibody varies inversely as the concentration of the drug under investigation. These methods have not been used in this study and will not be discussed further.

1.4 ANALYSIS OF DRUGS AND METABOLITES IN BODY FLUIDS BY NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

1.4.1 GENERAL

Analysis of biological fluids present special problems in NMR spectroscopy, i.e.:

i) a large signal arising from water which limits the dynamic range of the spectrum and makes observation of small resonances nearby difficult.

ii) line-broadening arising from solutions with high viscosity, binding to immobile macromolecules and membranes, the macromolecules themselves and the presence of paramagnetic ions.

iii) complex spectra due to the multicomponent nature of body fluids requiring good resolution for identification and quantification of signals.

The use of high magnetic fields and FT instrumentation together with on-line computer manipulation of the spectra has overcome some of the above problems (see section 1.3.3.2).

Intact urine gives minor problems when compared to other biological samples because it is less viscous and has a low content of macromolecules. A large number of well resolved signals from mobile protons of low molecular weight metabolites in urine have been obtained from NMR spectra[92-95]. Information yielded by ^{1}H NMR monitoring of metabolic alterations can be used to trace toxic and metabolic disorders in clinical pathology. In the case of diabetes mellitus, the concentration of glucose in urine can be determined[92,96] without interference from other substances such as ascorbic acid and carbohydrates[92]. A large phenylalanine peak was observed in the urine from patients with phenylketonuria[97]. Furthermore, the ratio of aromatic amino acids to branched amino acid measured by ^{1}H NMR[97] in urine or serum from patients has proved a helpful test for determination of the severity of hepatic failure. ^{1}H NMR has also proved valuable for the diagnosis of certain human metabolic disorders[98] and inborn errors of metabolism, especially the organic acidurias[99-101] such as propionic acidemia, methylmalonic aciduria, glutaric aciduria type I and isovaleric acidemia. Furthermore, an informative pattern was

revealed by ^{1}H NMR of associated biochemical changes derived from the abnormal patterns of metabolic excretion of mercury and cadmium induced nephrotoxicity of rats[102,103]. In this case, decreases in the excretion of creatinine and citrate, and increases in glucose, glycine, alanine, α-ketoglutarate, succinate and acetate were found from the ^{1}H NMR urinary metabolite "fingerprints" and quantitative analysis of the spectra. Thus a correlation could be drawn between histopathology and enzyme induction data. In addition to examination of endogenous compounds, metabolites excreted in urine from relatively high therapeutic dosage drugs have been detected and quantified simultaneously, e.g. paracetamol[94,104-106], oxpentifylline which is used in the treatment of vascular disease[107] and metronidazole[108]. Furthermore, NMR can provide information on previously unknown metabolic and excretory mechanisms of drugs, e.g. N-methylformamide in rats and mice[109-110].

NMR can also be used to examine endogenous and drug metabolites in blood, serum and plasma of human and experimental animals[111-114]. Measurement of lactic acid in serum with ^{1}H NMR spectroscopy[115] has been suggested as a possible criterion for the diagnosis of cancer in humans. It was found that 87% of the serum specimens from patients with various cancers yielded a proton signal ascribed to the methyl protons of lactic acid, whereas only 9% of serum specimens from normal controls did so. In tracing diabetic ketoacidosis by ^{1}H NMR, ketone-bodies (acetone, acetoacetate and β-hydroxybutyrate) from serum of patients were found to be larger than in control serum[111,113]. Raised levels of plasma ethanol and acetate in alloxan-induced diabetic rats[116] were also found using ^{1}H NMR. The normal ^{1}H NMR spectra of blood,

serum, plasma, erythrocytes and hepatocytes are too poorly resolved to be interpreted readily. However, multiple pulse sequences that generate "spin-echoes" permit spectra of small, rapidly tumbling molecules to be recorded without interference from the proton resonances of slowly moving macromolecules[117] (see section 1.3.3.2.). Normal and abnormal metabolites in body fluids were readily resolved in spin-echo ^{1}H NMR detection[103,112,117-119]. Quantitation of spin-echo spectra should be done with caution due to some intensity distortions of the peaks because of phase modulation (see section 1.3.3.2). In insulin-dependent diabetics, detection of mobile fatty acids as well as build-up of ketone bodies were observed in plasma using high-resolution spin echo ^{1}H NMR[112]. There was also a significant reduction in plasma alanine and elevation of valine that could be related to metabolic changes caused by insulin deprivation. In the case of the non-insulin dependent diabetics with hypertriglyceridaemia, although glucose was clearly elevated there was no ketoacidosis, and in addition there were very large signals from mobile triglycerides[113]. Glutathione, lactate, pyruvate, and some residues of haemoglobin can also be detected, and the production rate of lactate and of the reduction of oxidised glutathione upon addition of glucose to the erythrocyte suspension can be measured[117].

In some cases it is necessary to apply preliminary extraction procedures to achieve sufficient sensitivity for analysis or to isolate compounds of interest whose resonances are not completely resolved in the intact body fluid. Preliminary clean-up of samples can be done by acid extraction, solvent extraction or column chromatography. In a solvent or acid extract, proteins are left behind and their broadening effect is eliminated, with the result

that twelve amino acids and 19 other intermediary metabolites could be measured quantitatively with a single ^{1}H NMR spectrum of a cytosolic extract[120]. Free and esterified cholesterol have been quantified separately in methanol extracts of plasma[112]. Some "uraemic middle molecules" have been discovered in the plasma of uremic patients and the urine of healthy subjects by proton and carbon resonances after isolation with gas, anion-exchange and high performance liquid chromatography[121]. Solid phase extraction (using Bond Elut^{R} columns) has been used to separate urinary metabolites of the non-steroidal anti-inflammatory drugs, naproxen[122] and ibuprofen[123]. Another way to achieve more sensitivity from very low concentrations of compound is lyophilization[106,107,122] before redissolution in a suitable solvent.

A technique which aids assignment of spectra arising from complex mixtures is two-dimensional (2D) NMR[46-47]. Various types of 2D experiments have been developed and can be roughly divided into those which correlate chemical shifts (e.g. proton-proton or proton-carbon) and those which resolve coupling patterns. Proton-proton chemical shift correlation (COSY) has been applied to a urine sample concentrated by lyophilization from a person who ingested 1g of paracetamol and provided confirmation of previous assignments of 1D-spectra[124].

Several nuclei other than protons have been applied in metabolism studies. The most developed are ^{13}C, ^{31}P and ^{19}F NMR spectroscopy. Unlike the ^{1}H NMR spectrum, which has chemical shift range of 10 ppm, the ^{13}C NMR spectrum's range is about 200 ppm, thus individual peaks are more easily resolved. However ^{13}C NMR is inherently less sensitive than of proton since ^{13}C nuclei have a

natural abundance of only 1.1% and a weaker magnetic moment. The reduced sensitivity limits the biomedical applications of ^{13}C NMR spectroscopy in some ways. However labelling compounds with ^{13}C nuclei, makes it possible to study in vitro metabolism (see section 1.5.1). Interest in ^{31}P NMR developed rapidly for three main reasons, (a) the 100% natural abundance of ^{31}P; (b) the number of cytosolic metabolites that contain ^{31}P is much smaller than those that contain ^{1}H and ^{13}C, thus usable resolution is easy to achieve; and (c) the phosphorylated compounds that can be detected in biological tissues by NMR are intimately involved in energy metabolism. A single ^{31}P NMR spectrum provides a quantitative measure of sugar and nucleoside monophosphates, inorganic phosphate, phosphocreatine, nucleoside di- and triphosphates, including ADP and ATP, and cofactors such as NAD. It can also be used to measure pH and free magnesium concentration in erythrocytes or other living cells. ^{31}P NMR has been used to characterize the phosphorus metabolism of various healthy and diseased biological materials[125-129]. Another nucleus used in metabolism studies is ^{19}F which has 100% natural abundance and a relative sensitivity of 0.83 to that of proton. Despite its lack in most biological molecules, ^{19}F spectroscopy can be used to monitor the metabolism of fluorinated drugs in biological fluids without interference from endogenous compounds[130]. Flucloxacillin[130], 5-fluorouracil[131-132], and fluoropyrimidine[133-136], together with their metabolites, have been detected in urine or plasma. The possibility of simultaneous observation of free as well as plasma protein-bound fluoropyrimidine demonstrated the suitability of the ^{19}F NMR procedure for direct drug-protein interaction studies[136].

1.4.2 MODEL DRUGS

Four model drugs with known metabolic pathways, i.e. aspirin, paracetamol, acemetacin and D-penicillamine, were chosen for drug metabolism studies using ^{1}H NMR spectroscopy and/or spectrophotometry and HPLC. The relatively high concentrations and rapid excretion of aspirin and paracetamol in urine after overdose make them suitable candidates for quantitative study by ^{1}H NMR.

1.4.2.1 ASPIRIN (ACETYLSALICYLIC ACID)

Aspirin (ASA) is a mild analgesic effective against peripheral pain of low to moderate intensity[137], with antipyretic[138,139], and anti-inflammatory[140,141] properties. It has been proposed that its primary clinical effect is related to inhibition of bradykinin and prostaglandin systems i.e. to the synthesis and release of these mediators[140].

Disposition and Metabolism of Aspirin

Aspirin is absorbed from the stomach and small intestine. Absorption is rapid following oral administration of conventional tablets or capsules, but the rate is affected by gastric emptying time and the presence of food in the GI tract[142]. Although the presence of food in the stomach markedly delays the time to reach peak concentration of aspirin, there is no significant difference in the amount absorbed[143].

Aspirin is rapidly hydrolysed in the body to salicylic acid (SA) with a biological half-life of 15-20 minutes[143-144]. The drug is subject to hydrolysis by esterases during absorption in the intestinal wall[145-147] and in the liver[148]. In blood, the drug is also rapidly hydrolyzed mainly by red blood cell aspirin

esterase[149]. In therapeutic doses, both aspirin and salicylic acid are bound to plasma proteins, mainly albumin, and this accounts for 80-85%[150] or 90-95%[151,152] respectively of the total salicylate in plasma.

After aspirin is metabolised to salicylic acid in the intestinal wall, liver and blood, further metabolism of salicylic acid occurs primarily in the hepatic and renal endoplasmic reticulum and mitochrondria[141]. The metabolic fate of salicylic acid was first described by Kapp and Coburn (1942)[153]. The three main metabolites of salicylic acid (SA) are salicyluric acid (SUA, the glycine conjugate), the ether or phenolic glucuronide (SPG) and the ester or acyl glucuronide (SAG). Gentisic acid (GA), the minor metabolite, is formed by ring hydroxylation of salicylic acid. Recently, Zimmerman et al. (1981)[154] have characterised a double conjugate of SA, namely phenolic glucuronide of salicyluric acid (SUPG) in the body fluids of uraemic patients. Also, small amounts of gentisuric acid are formed by glycine conjugation of gentisic acid or hydroxylation of salicylic acid[155]. The main metabolic pathways of aspirin are outlined in figure 1.4.1.

Plasma half-life of salicylic acid varies from about 3 h in analgesic doses to 20 h or more at anti-inflammatory and toxic doses[156]. Thus, increasing the dose without increasing the interval between doses may cause accumulation and toxic effects. In the body, the major route of salicylic acid elimination is via renal excretion of both salicylic acid and metabolites. Following a single oral dose of aspirin (1 g) in four healthy volunteers, Hutt, Caldwell and Smith (1982)[157] found a mean recovery of 96.2% of administered dose in 0-24 h urine. The major urinary metabolite was SUA (63.9%); the remainder of the excreted dose comprised GA

(1.3%), SPG (5.9%), SAG (4.0%), SUPG (6.2%) and SA (13.5%). Urinary pH can profoundly alter the proportion excreted as free SA[158,159].

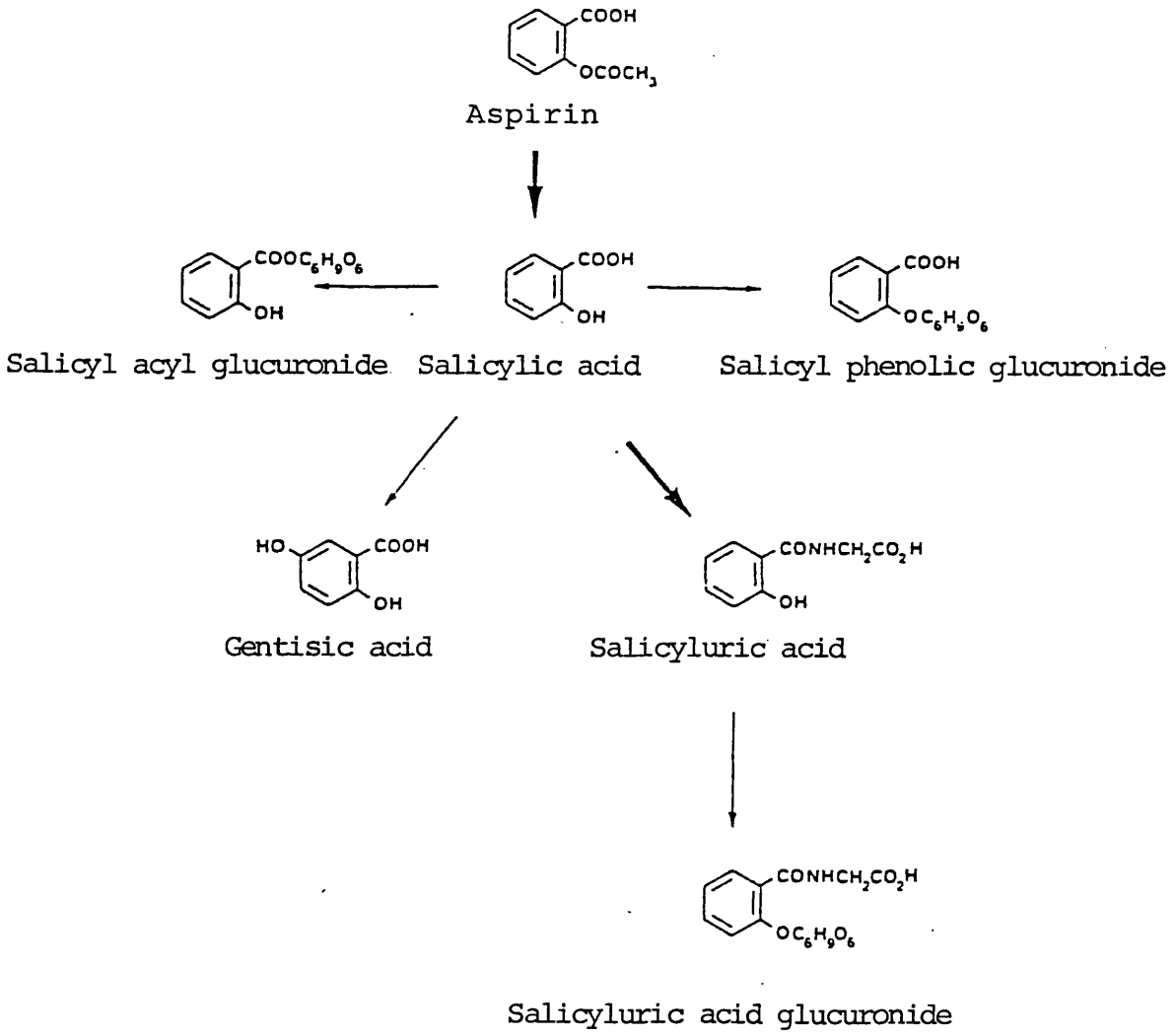


Fig. 1.4.1 Metabolic pathways of aspirin

The renal clearance of the drug increases almost exponentially with increase in pH above 5.5-6.5. The metabolism of salicylic acid is dose dependent due to the limited capacity formation of two primary metabolites, namely salicyluric acid and salicyl phenolic glucuronide[160]. Levy, Tsuchiya and Amsel (1972)[161] suggested that the liver was limited in its ability to form these conjugates. Hence, as the dose is increased, the relative proportion of the minor metabolites, including salicylic acid, also increases.

Toxicity of Aspirin

Serious adverse reactions occur infrequently with usual analgesic doses (0.6-1g). Gastrointestinal symptoms such as gastric distress, heartburn or nausea may occur after therapeutic doses, particularly in the elderly. Recently, Rees and Turnberg (1980)[162] concluded that although acute exposure of the gastric mucosa result in increased blood-loss, abnormal mucosal function and short lasting morphological damage, there was little evidence that low dose aspirin consumption results in either peptic ulcer or acute gastrointestinal bleeding.

High doses of aspirin may cause tinnitus, deafness, nausea, abdominal pain, respiratory alkalosis[163] metabolic acidosis[164,165] hyperglycaemia[166], and hypoprothrombinemia[167,168]. Blood salicylate concentrations are used to assess the degree of toxicity. These concentrations are also frequently used in conjunction with the Done nomogram (fig.1.4.2) for estimating the severity of toxicity from acute salicylate overdose[169]. Unfortunately it appears that this nomogram cannot be used in chronic salicylate intoxication and in cases of poisoning from

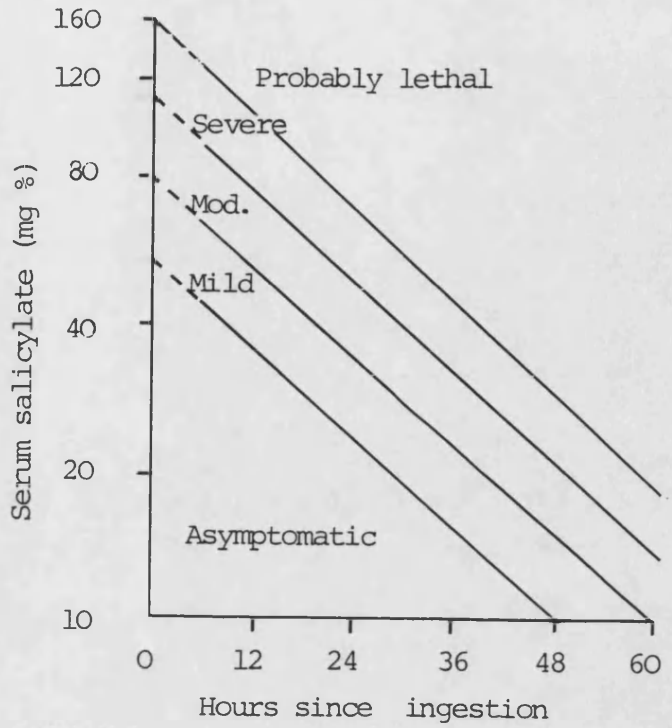


Fig. 1.4.2. The Done nomogram for estimating severity of acute salicylate toxicity

sustained-release aspirin preparations because of the delayed release.

In chronic use, salicylates vary in their propensity to induce gastric ulceration and haemorrhage in man and laboratory animals[141]. Aspirin may have nephrotoxic[170] and hepatotoxic[154] effects, but these usually occur in association with diseases such as rheumatic fever, juvenile rheumatoid arthritis and systemic lupus erythematosus when aspirin is taken in large doses over a long period.

1.4.2.2 PARACETAMOL (ACETAMINOPHEN)

Paracetamol(P), an analgesic and antipyretic drug, is used in mild to moderate pain which is not of visceral origin and which is not accompanied by significant inflammation. Its antipyretic

effect may stem from the inhibition of endogenous pyrogens on the hypothalamic heat-regulating centres[138] and its analgesic effect from the inhibition of prostaglandin synthesis.

Disposition and Metabolism

Paracetamol is rapidly and almost completely absorbed from the gastrointestinal tract following oral administration. Its peak plasma concentration occurs 30-60 minutes after ingestion of the usual analgesic dose of 0.5-1 g. However, a correlation between serum level and analgesic effect has not been demonstrated[171]. In addition, gastric emptying time is a major variable in determining the rate but not the extent of paracetamol absorption. Distribution of paracetamol is relatively uniform and it is distributed throughout most body fluids. Although protein binding appears to be insignificant at therapeutic concentrations, it does increase to about 20% when the drug is present in toxic concentrations[172].

Paracetamol is metabolised in the liver largely (60-90%) as a result of conjugation with glucuronic acid (PG) and sulphate (PS). In addition, a small amount of paracetamol (5-10%) is oxidised by the cytochrome P-450-dependent drug-metabolising enzyme(s) to a reactive intermediate(s). This intermediate is preferentialy conjugated with hepatic glutathione and after further metabolism to paracetamol cysteine (PC) and paracetamol mercapturic acid (PMA) it is excreted in the urine[173]. Hepatic glutathione, however, is limited and once it is depleted covalent binding to hepatocytes may occur resulting in cell death, and possibly irreversible hepatotoxicity. The metabolic pathways of paracetamol[174] can be outlined as in fig. 1.4.3.

Following metabolism, paracetamol is mainly eliminated by the

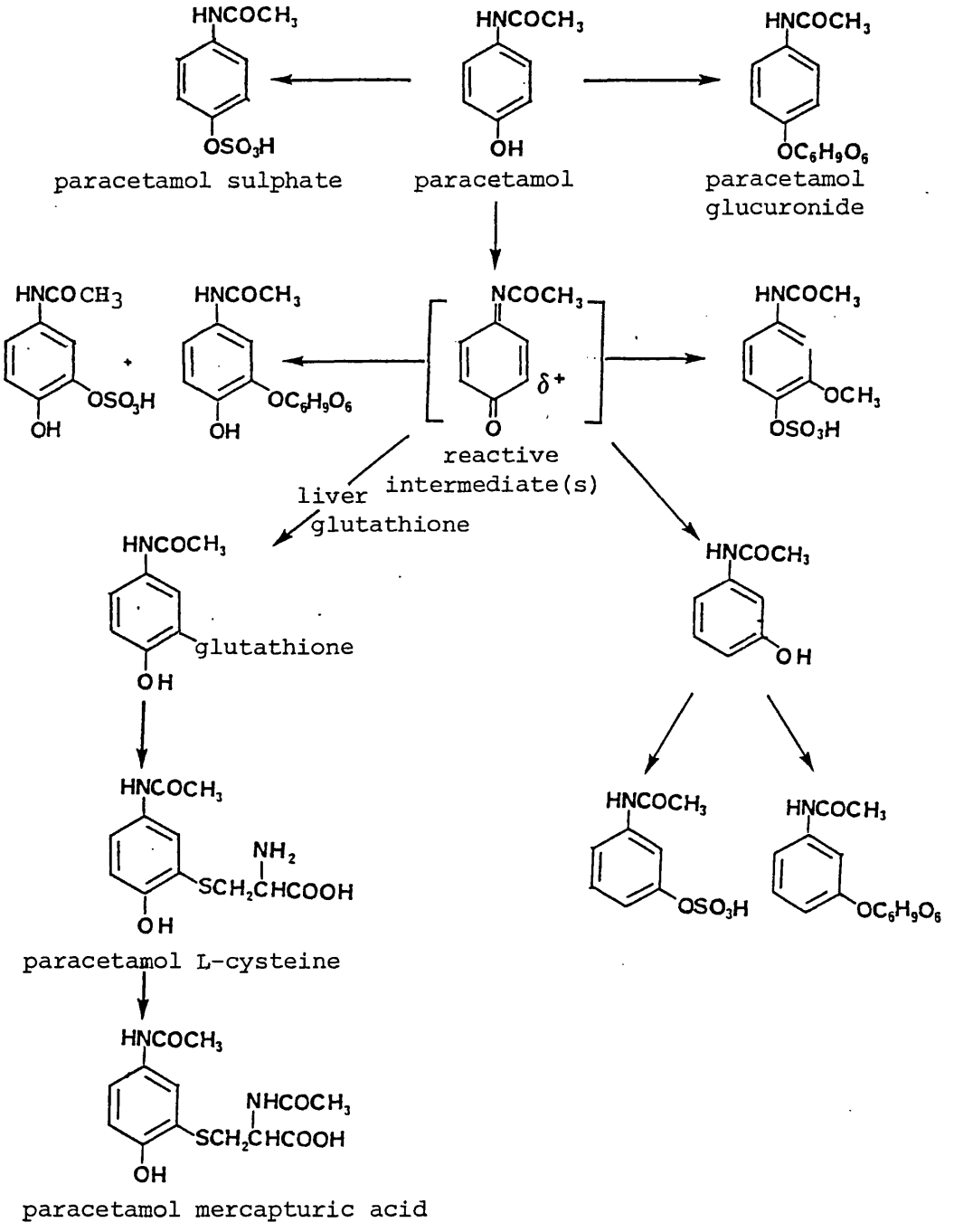


Fig. 1.4.3 Metabolic pathways of paracetamol

kidneys with a mean plasma elimination half life of about 3 h[171] and only a small amount (about 5%) is excreted unchanged in urine. In patients with impaired renal function, the conjugated metabolites accumulate in the blood but the unchanged drug does not[175].

Toxicity

Unlike aspirin, paracetamol does not apparently cause gastric mucosal injury. Adverse reactions occur infrequently and hypersensitivity only rarely. Paracetamol is a metabolite of phenacetin and acetanilide, but unlike these drugs, produces little or no methaemoglobinaemia and reports of haemolytic anaemia have been rare. Serious toxicity with paracetamol was first recognised in 1966 when fatal hepatic necrosis after overdosage was reported[176,177]. Early symptoms of toxicity include nausea, vomitting, diarrhoea, diaphoresis, pallor and abdominal pain.

The biochemical mechanism of the hepatotoxicity of paracetamol was first suggested by Mitchell et al. in 1973[178]. Studies on the mechanism of paracetamol-induced liver damage in laboratory animals showed that paracetamol is converted in the body to a chemically reactive arylating agent that covalently binds to vital hepatocellular macromolecules. In addition a direct relationship was demonstrated between the formation of a paracetamol glutathione conjugate, arylation of hepatic macromolecules and hepatic damage after paracetamol ingestion. The likelihood of damage occurring after an overdose of paracetamol may be predicted by measurement of the plasma paracetamol level[176] as expressed in fig. 1.4.4. Despite the effect on the liver of acute overdose of paracetamol, there have been only a few reports of hepatotoxicity arising as a result

of chronic ingestion[154,179].

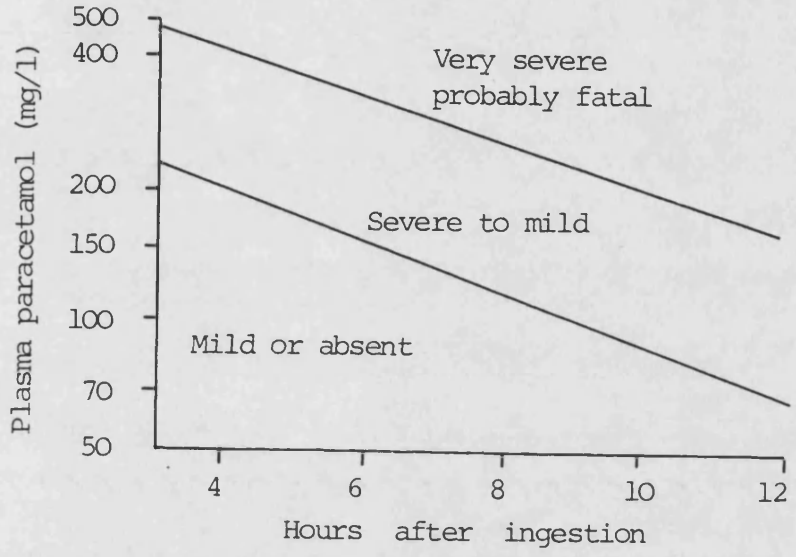


Fig. 1.4.4 The degree of toxicity after an overdose of paracetamol related to plasma concentrations and time after ingestion

1.4.2.3 ACEMETACIN

Acemetacin (ACE) is a new non-steroidal anti-inflammatory drug (NSAID) of the indole group with analgesic and antipyretic effects. This drug is a glycolic acid ester derivative of the NSAID indomethacin (IN). It was synthesised at the Research Laboratories of Troponwerke, Federal Republic of Germany, and selected out of several hundred indole derivatives for further clinical

investigations because of its anti-inflammatory activity and low ulcer genicity in animal models[180]. In clinical studies it has been shown to provide symptomatic relief in moderate to severe rheumatoid arthritis (RA)[181]. It is now on general release in Federal Republic of Germany, and currently being considered by the Committee for Safety of Medicines (CSM) for use in the U.K.

Disposition and Metabolism

There have been several clinical studies on the metabolism of acemetacin, and its metabolic pathway[181] is given in fig. 1.4.5. Following chronic dosing of 180 mg/day acemetacin for 10 days in 12 RA patients, the mean steady state blood level of acemetacin plus indomethacin was 1.85 μM[181]. It was also found that approximately 50% of an orally administered dose of acemetacin appeared in the serum as indomethacin. In human serum dose-dependent protein binding in the range of 81.5-93% was observed with acemetacin[181].

The metabolites of acemetacin were found to be similar to those of indomethacin[181] (see fig. 1.4.5). Following absorption and distribution of acemetacin in man, it is excreted as the unchanged drug, indomethacin, and their glucuronide-conjugates. O-demethylation may also occur and the products of this degradation, desmethylindomethacin and desmethylacemetacin, are excreted in both free and glucuronide-conjugated forms. Deacylation occurs and the free and conjugated products, des-p-chlorobenzoyl indomethacin and des-p-chlorobenzoyl acemetacin, are excreted in urine. In addition, experiments in rats[182] have also shown indomethacin glycine conjugate which was excreted renally in approximately three times greater concentration than it was after indomethacin administration. The mean half life

of acemetacin following the last acemetacin dose (180 mg/day for 10 days) in RA patients was 5.22 h and that of indomethacin plus acemetacin 4.52 h[181].

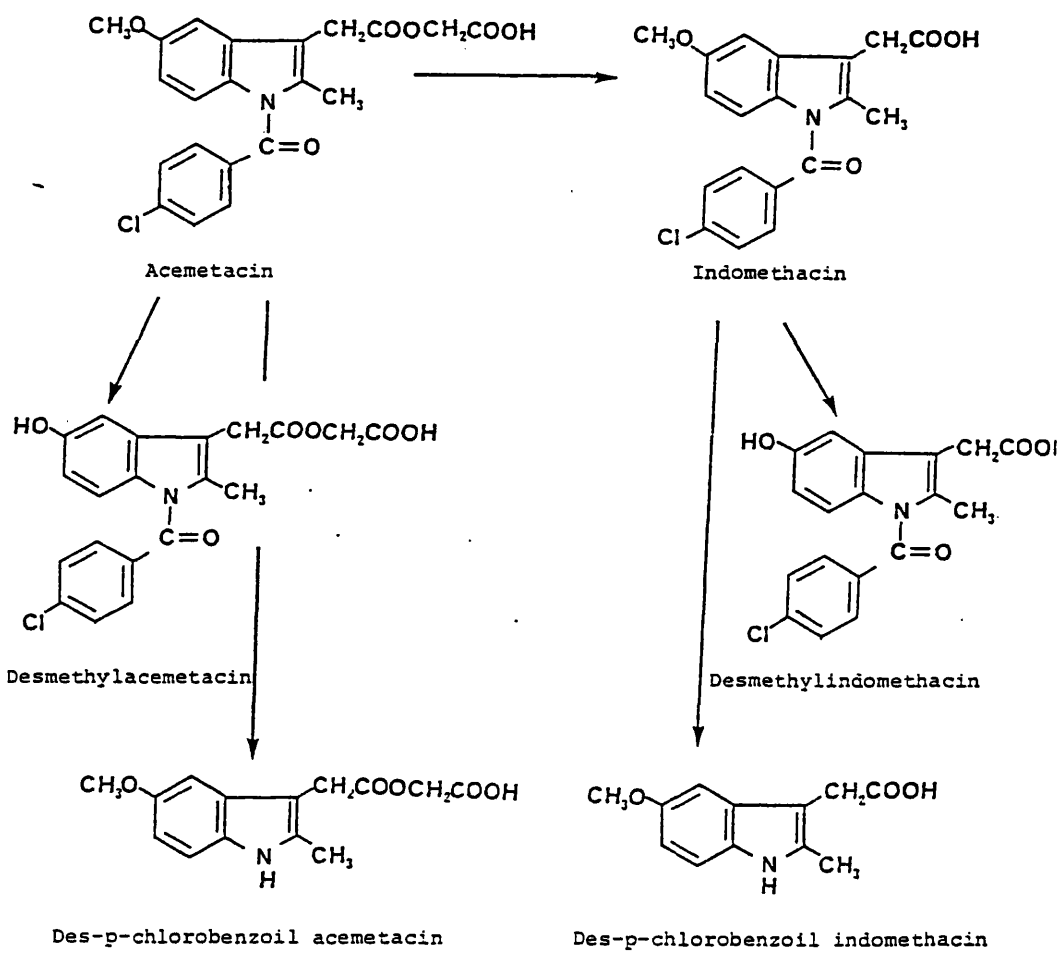


Fig. 1.4.5 Metabolic pathways of acemetacin

1.4.2.4 D-PENICILLAMINE

Penicillamine (PSH), was first identified by Abraham and colleagues[183] in 1943 as a penicillin-hydrolysis product during studies to elucidate the structure of penicillin. D-Penicillamine is a thiol-containing amino acid, used clinically for Wilson's

disease[184], cystinuria[185], rheumatoid arthritis (RA)[186], lead[187] and mercury poisoning[188] and morphoea[189]. In spite of RA being the commonest condition treated with D-penicillamine, the pharmacology and mode of action of this drug in RA is still not completely understood. In the treatment of RA, D-penicillamine appears to be a valuable drug, particularly in severe seropositive RA with visceral complications[190], in which this agent dissociates macroglobulin rheumatoid factor[191] and has immunosuppressive activity to the etiologic stimulus[192].

D-Penicillamine is rapidly absorbed from the intestine after oral administration[193]. The peak plasma concentration of total D-penicillamine following a single dose was 60 μM at between 2 to 4h[194]. Its absorption was significantly higher when the drug was given before food or during fasting than when taken with food[195]. The metabolism of D-penicillamine is complex; it is known to exist in 5 forms in physiological fluids, excluding a protein-bound fraction (see fig. 1.4.6). The only metabolically-transformed metabolite of D-penicillamine identified is S-methyl-D-penicillamine (MPSH) which is methylated in the liver[195]. On the basis of several experiments it appears that some 80% of D-penicillamine present in plasma is protein-bound, around 7% is in the form of D-penicillamine L-cysteine disulphide (PSSC), apprximately 6% free D-penicillamine (PSH) and 5% D-penicillamine disulphide (PSSP). The final 2% is unaccounted for and could be in the form of methylated metabolites, other disulphides or metal complexes[195].

The percentage of each urinary metabolite appears to vary according to the disease being treated. All 5 forms of D-penicillamine metabolites (fig. 1.4.6) are known to be excreted in

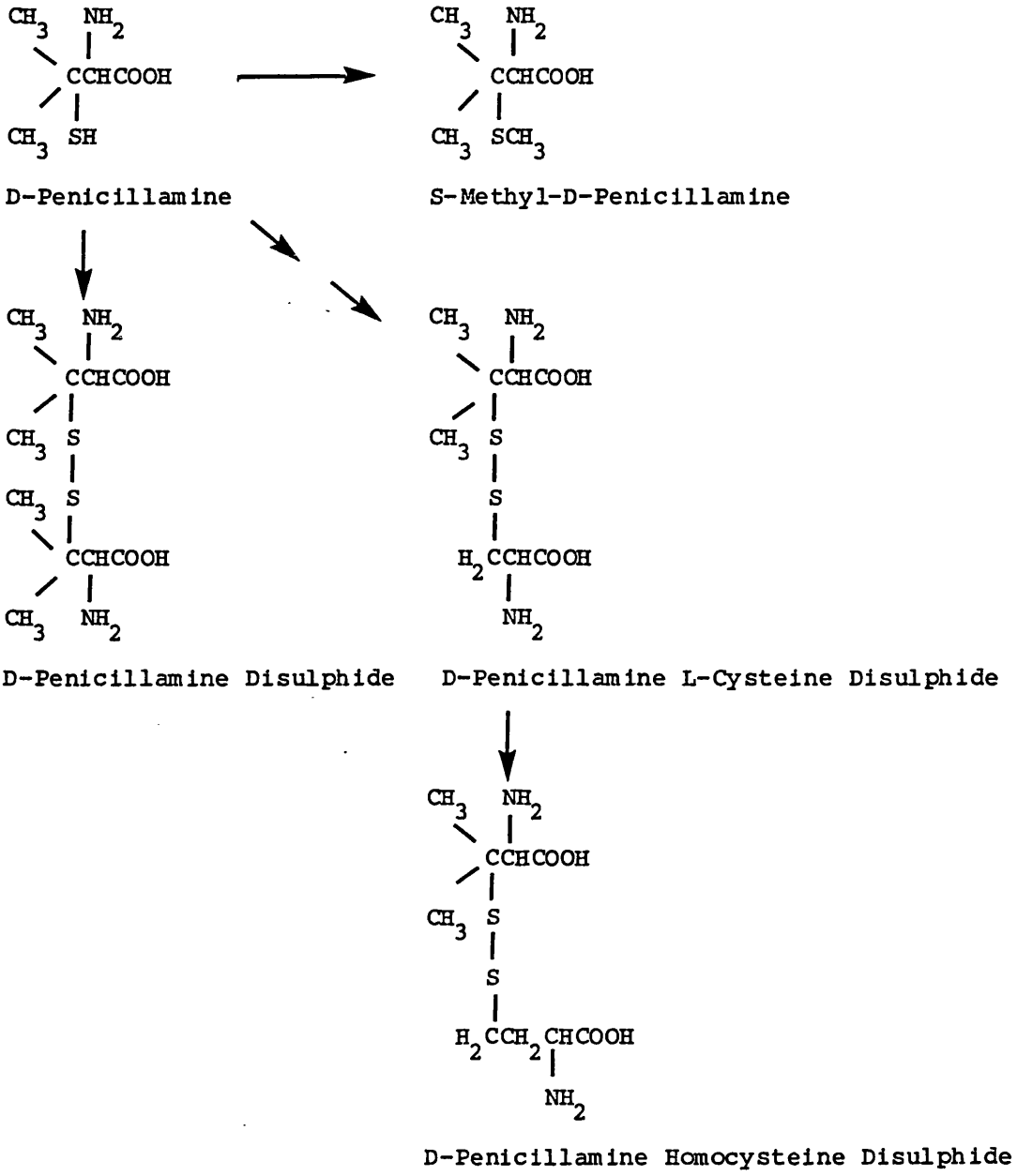


Fig. 1.4.6 Metabolic pathways of D-penicillamine

the urine of cystinuric, Wilson's and RA patients. D-penicillamine homocysteine disulphide was excreted in excess in Wilson's disease patients and S-methyl-D-penicillamine in RA patients[195]. In an experiment using ^{14}C-labelled D-penicillamine in rats, a biphasic loss of plasma label occurred[196,197] and similar results have been obtained from studies which measured total D-penicillamine in human volunteers, showing a rapid elimination phase (half life about 1-5 h) and a slower phase. Estimates of the half life of the second slower phase vary from 4-6 days[198] to 8 days[199].

Analysis of D-penicillamine and its metabolites in biological fluids is difficult due to the high reactivity of the SH group. D-Penicillamine has been assayed using a variety of methods such as colorimetric and spectrophotometric assays[200,201], ion-exchange chromatography[202], gas chromatography[203] radioimmunoassays[204,205], titration using ion-selective electrodes[206], high performance liquid chromatography[207-209] and amino acid analysis[210], but none have proven to be suitable for routine analytical use.

1.5 IN VITRO DRUG METABOLISM STUDIES BY NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

1.5.1 GENERAL

In vitro drug metabolism can be studied by NMR after incubation of a drug (i.e. substrate) with the enzyme system. The loss of substrate(s) and formation of product(s) can be measured simultaneously. Some examples of the results that can be obtained with ^{1}H NMR spectroscopy are the measurement of enzyme kinetics in crude cytosolic extracts[211], and the detection of a "new" metabolite of penicillin as an in vitro product of the incubation with serum albumin[212] which was subsequently observed in vivo. Hepatocytes, as the main site of metabolism in the body, have been used in in vitro studies. ^{1}H NMR studies of intact hepatocytes, isolated from rat liver, and cell extracts have together provided considerable insight into the metabolism of paracetamol[213]. Although it was known that these cells metabolised paracetamol, a spin-echo experiment after the incubation did not detect any metabolites. However, when the cells were lysed or in cell free extract, the presence of metabolites was confirmed. In addition, the mobile fatty acids in cellular suspensions of viable hepatocytes were detected with high resolution ^{1}H NMR. Other enzyme systems which have been used to study drug metabolism and detoxification are purified isozymes, cytochrome P-450 and other cytochromes from experimental animal livers. 18,19-dehydroxydeoxy-corticosterone, produced from 18-hydroxydeoxy-corticosterone incubated with cytochrome P-450 was identified by ^{1}H NMR[214]. A relationship between the binding and the oxidation of paracetamol[215] and aminopyrine[216] by rat liver cytochrome P-450

using longitudinal relaxation (T_1) measurements has also been observed. Data obtained on specific complex formation between these substrates and different cytochrome P-450's was found to be in agreement with the specificity of the enzymatic activity exhibited in metabolic studies.

The substrate under study may need special labelling if NMR insensitive nuclei are to be observed, e.g. ^{13}C. Such labelling has made it possible to characterize biosynthetic pathways of glucose[217] and formaldehyde[218] by Escherichia coli. Gluconeogenesis, glycogenolysis and lipogenesis have also been observed directly by ^{13}C NMR of perfused rat liver or isolated hepatocytes[219-222]. The metabolism of ^{13}C labelled aminopyrine by a perfused liver system has been used to evaluate the hepatic drug metabolising enzyme activity before and after induction with phenobarbitone[223]. Studies of in vitro metabolism have also been done using other NMR active nuclei such as ^{31}P and ^{19}F. The phosphorylation status of rat liver by ^{31}P NMR spectroscopy and its implications for metabolic control in vitro[220,224] and in vivo[220] have been reported. The incorporation of 5-fluorouracil into RNA and its conversion to metabolites in intact Escherichia coli cells has been followed by ^{19}F NMR[225]. Although these studies involve only animal models, they provide insight into the further development of such measurements.

1.5.2 MODEL SUBSTRATES

Cytochromes P-450 and P-448 may be induced in vivo by exogenous agents as discussed in section 1.2.2. It is possible that NMR may be used to distinguish which cytochrome has been induced by following the metabolism of model substrates utilizing these

enzymes. Thus a substrate metabolised primarily by cytochrome P-450 would have its rate of metabolism increased following induction of P-450 but not if the P-448 level was increased. In the present study, NMR has been used to follow the metabolism of two model substrates, i.e. aminopyrine (metabolised by cytochrome P-450) and daunorubicin (metabolised by cytochrome P-450 and P-448), after enzyme induction using phenobarbitone (PB) and 3-methylcholanthrene (3MC) as cytochrome P-450 and P-450/P-448 inducers respectively.

1.5.2.1 AMINOPYRINE

Aminopyrine (4-dimethylaminoantipyrine, DMAP) has analgesic, anti-inflammatory and antipyretic actions, but owing to the risk of agranulocytosis its use is discouraged[226]. Aminopyrine undergoes two N-demethylations by hepatic microsomal enzymes to form first 4-monomethylaminoantipyrine (MMAP) and then ampyrone (4-aminoantipyrine, AP)[32,227] as shown in fig. 1.5.1. Aminopyrine is a model substrate to study N-dealkylation of drugs and thus is sometimes used in drug metabolism studies.

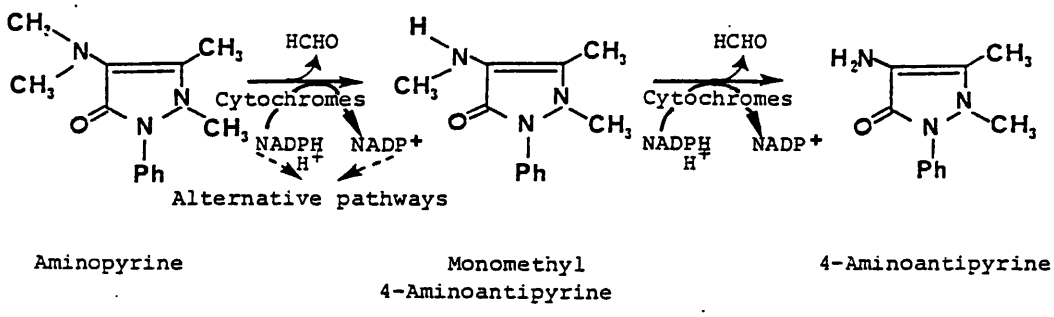


Fig. 1.5.1 The metabolic pathways of aminopyrine

1.5.2.2 DAUNORUBICIN

Daunorubicin (DA) is an anthracycline antibiotic for the treatment of acute nonlymphocytic leukemia in adults[228]. Human metabolism of daunorubicin involves carbonyl reduction, reductive glycosidic cleavage, O-demethylation, O-sulphation and O-glucuronidation. Its metabolites in human urine have been identified as daunorubicinol, daunorubicinol aglycone, deoxydaunorubicin aglycone, deoxydaunorubicinol aglycone, demethyl deoxydaunorubicinol aglycone, deoxydaunorubicinol aglycone 13-O-β-glucuronide, demethyl deoxydaunorubicinol aglycone 4-O-sulphate and demethyl deoxydaunorubicinol aglycone 4-O-β-glucuronide[229]. In rat tissues and homogenates, daunorubicin was found to be metabolised extensively to daunorubicinol by a soluble enzyme in the cytosol, and their aglycones, daunorubicin aglycone and daunorubicinol aglycone respectively, by liver microsomal enzymes[230]. The pathway for daunorubicin metabolism in rat liver fractions[230] is shown in fig. 1.5.2.

1.6 SCOPE OF THE THESIS

The present study aims to highlight some areas of ^{1}H NMR spectroscopy applied to drug metabolism studies. The aims are to assess the capabilities and limitations of ^{1}H NMR in the analysis of body fluids, such as urine and plasma, and rat liver preparations. NMR methods for the determination of aspirin, paracetamol, acemetacin and D-penicillamine in biological fluids will be developed, and the results obtained compared to those from more traditional analytical methods (HPLC, UV and visible spectroscopy). The capability of ^{1}H NMR as a tool for studying *in vitro* metabolism will be assessed using two model substrates, i.e.

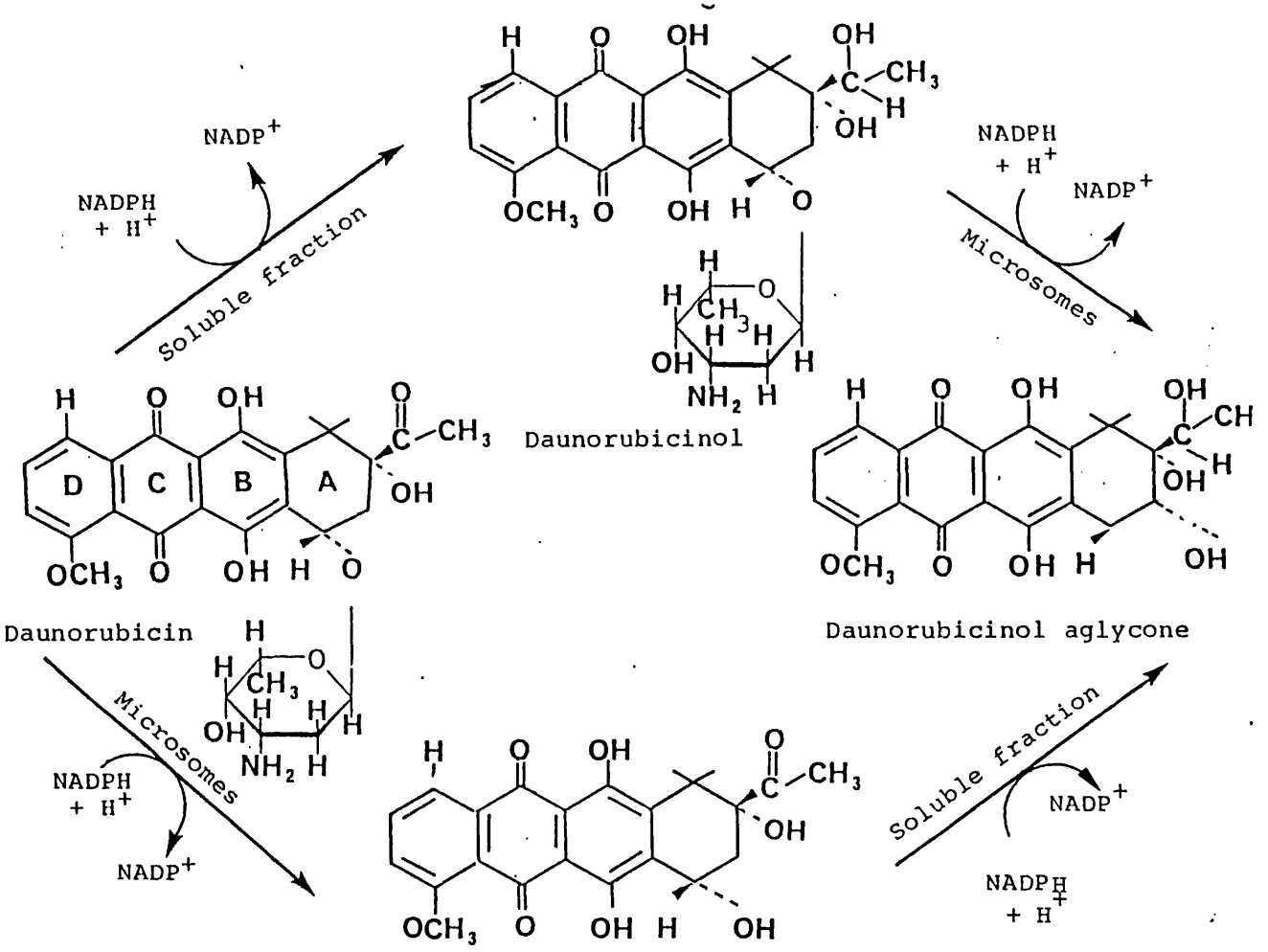


Fig. 1.5.2 Metabolic pathways of Daunorubicin in rat liver fractions

aminopyrine and daunorubicin, in 10,000 g liver fraction as a general site of metabolism.

The thesis is divided into five chapters:

- Chapter One has been devoted to an introduction on drug metabolism, NMR analysis of body fluids and *in vitro* metabolism. This chapter includes brief descriptions of the model drugs and substrates used in the present study.
- Chapter Two consists of material and methods.
- Chapter Three gives the results of drug analysis in body fluids using ^{1}H NMR and compares the results to HPLC and spectrophotometry data.
- Chapter Four gives the results for *in vitro* metabolism studies by ^{1}H NMR.
- Chapter Five discusses the application of ^{1}H NMR to drug metabolism studies.

CHAPTER TWO

MATERIALS AND METHODS

2.1 COMPOUNDS

a) Analytes

i) acemetacin (1-(p-chlorbenzoyl)-5-methoxy-2-methylindol-acetoxy-acetic acid), $C_{21}H_{18}ClNO_6$, MW = 415.8 (Troponwerke GMBH & Co. KG Cologne).

ii) aminopyrine (4-dimethylaminoantipyrine), $C_{13}H_{17}N_3O$, MW = 231.3 (Sigma Chemical Co.).

iii) ampyrone (4-aminoantipyrine), $C_{11}H_{13}N_3O$, MW = 203.2 (Sigma Chemical Co.).

iv) O-anisic acid (O-methoxy benzoic acid), $CH_3OC_6H_4CO_2H$, MW = 152.2 (Aldrich Chemical Co. Inc.).

v) daunorubicin ((8S-cis)-8-Acetyl-10[(3-amino-2,3,6-trideoxy - α -L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione) - hydrochloric acid, $C_{27}H_{29}NO_{10}.HCl$, MW = 564.0 (May & Baker Ltd.).

vi) gentisic acid (2,5-dihydroxybenzoic acid), sodium salt, $C_7H_5O_4Na$, MW = 176.1 (Sigma Chemical Co.).

vii) indomethacin (1-p-chlorbenzoyl)-5-methoxy-2-methylindol-3-acetic acid), $C_{19}H_{16}ClNO_4$, MW = 357.8 (Sigma Chemical Co.)

viii) methionine (2 - amino - 4 - (methylthio) - butyric acid), $CH_3S(CH_2)_2.CH(NH_2).COOH$, MW = 149.2 (BDH Chemicals Ltd.).

ix) paracetamol B.P. (N-acetyl-4-aminophenol), $C_8H_9NO_2$, MW = 151.2 (Pharmacy, Royal United Hospital, Bath).

x) paracetamol glucuronide (4-hydroxy-N-phenylacetamide-4-glucuronide), $C_{14}H_{17}NO_8$, MW = 327.3 (kindly donated by Stirling Winthrop, R & D).

xi) paracetamol sulphate (4-hydroxy-N-phenylacetamide-4-

sulphate), $C_8H_9NSO_5$, MW = 231.2 (kindly donated by Sterling Winthrop, R & D).

xii) D-penicillamine (dimethylcysteine), $(CH_3)_2C(SH).CH(NH_2).COOH$, MW = 149.2 (Sigma Chemical Co.).

xiii) phenobarbitone (5-ethyl-5-phenyl-2,4,6-trioxohexahydropyrimidine), sodium salt, MW = 254.2 (May & Baker Ltd.).

xiv) S-methyl penicillamine, $(CH_3)_2C(SCH_3).CH(NH_2).COOH$, MW = 163.2 (kindly donated by Lilly Research Centre Ltd.).

xv) D-penicillamine disulphide (3,3,3',3'-tetramethylcystine), $((CH_3)_2CS.CH(NH_2).COOH)_2$, MW = 296.4 (Fluka AG).

xvi) salicylic acid (2-hydroxy benzoic acid), sodium salt, $C_6H_4(OH).COONa$, MW = 160.1 (Sigma Chemical Co).

xvii) salicyluric acid (O-hydroxy hippuric acid), $C_9H_9NO_4$, MW = 195.2 (Sigma Chemical Co.).

xviii) TPS (3-(Trimethylsilyl)-1-propane-sulphonic acid), or DSS (2,2-dimethyl-2-silapentane-5-sulphonate), sodium salt, 99+%, MW = 236.3 (Aldrich Chemical Co. Inc.).

xix) L-valine (2-aminoisovaleric acid), $(CH_3)_2CHCH.NH_2.COOH$, MW = 117.1 (Sigma Chemical Co.).

b) Solvents

i) acetone-d_6-99.5 atom %D, CD_3COCD_3, MW = 64.1 (Aldrich Chemical Co. Inc.).

ii) deuterium oxide - 99.8 atom %D, D_2O, MW = 20.0 (Aldrich Chemical Co. Inc.).

All other solvents were ANALAR grade or in the case of HPLC analysis, HPLC grade (Fisons).

c) Other Chemicals

i) acetoacetic acid, lithium salt, $CH_3COCH_2COO.Li$, MW = 108.0 (Sigma Chemical Co.).

ii) L-alanine (isolated from natural source), $CH_3.CH(NH_2).COOH$ MW = 89.1 (Nutritional Biochemicals Co.).

iii) creatine phosphate, disodium salt, $C_4H_8N_3O_5PNa_2.6H_2O$, MW = 363.2 (Boehringer Manheim GMBH).

iv) creatinine standard - clinical reagents 10.0 mM (in 0.1M hydrochloride acid), $C_4H_7N_3O$ (BDH Chemicals Ltd).

v) L-cysteine (2-amino-3-mercaptopropanoic acid), hydrochloride acid, $C_3H_7NSO_2.HCl$, MW = 157.6(BDH Chemicals Ltd.).

vi) Folin & Ciocalteau's reagent (Fisons).

vii) D(+)galactose, MW = 180.2 (May & Baker Ltd.).

viii) D(+)glucose, MW = 180.2 (May & Baker Ltd.).

ix) glucose-6-phosphate (D-glucose-6-dihydrogen phosphate), $C_6H_{13}O_9P$, MW = 260.1 (Sigma Chemical Co.).

x) glucurase enzyme (β-glucuronidase or β-D-glucuronide glucuronoso hydrolase from bovine liver), buffered at pH = 5.0, 5000 units/ml (Sigma Chemical Co).

xi) D-glucuronic acid, $CHOH.(CHOH)_3.CH(O).COOH$, MW = 194.1 (BDH Chemicals Ltd.).

xii) glycine, $NH_2.CH_2.COOH$, MW = 75.1 (Fisons).

xiii) hippuric acid, $C_6H_5.CO.NH.CH_2.COOH$, MW = 179.2 (BDH Chemicals Ltd.).

xiv) L-histidine hydrochloride monohydrate, MW = 209.6 (sigma Chemical Co.).

xv) DL-lactic acid, $CH_3CHOH.COOH$, MW = 90.1 (Fisons).

xvi) L-leucine (2-amino-4-methylpentanoic acid), $(CH_3)_2CHCH_2CH$

(NH_2).COOH, MW = 131.2 (Nutritional Biochemicals Co.).

xvii) iso-leucine (2-amino-3-methylpentanoic acid), $CH_3CH_2CH(CH_3).CH(NH_2).COOH$, MW = 131.2 (Nutritional Biochemicals Co.).

xviii) mannitol, $CH_2(OH).(CHOH)_4.CH_2OH$, MW = 182.17(BDH Chemicals Ltd.).

xix) D-penicillamine hydrochloride (d-3-mercaptovaline HCl), $C_5H_{11}NSO_2.HCl$, MW = 185.7 (Sigma Chemical Co.).

xx) pyruvic acid, sodium salt, $CH_3CO.COONa$, MW = 110.0 (Sigma Chemical Co.).

xxi) sarcosine hydrochloride, $C_3H_7O_2N.HCl$, MW = 125.6 (Sigma Chemical Co.).

xxii) succinic acid, $(CH_2COOH)_2$, MW = 118.1 (BDH Chemicals Ltd).

xxiii) sulphatase enzyme (aryl sulphate sulfohydrolase from helix pomatia-type H5) 19800 units/g with β-glucuronidase activity (Sigma Chemical Co.).

xxiv) zerolit 325 - standard resin, cation exchange resin, functional group $R-SO_3-H^+$, from H^+, mesh 14-52 (BDH Chemicals Ltd.).

All other reagents were laboratory grade supplied by Sigma Chemical Co., BDH Chemicals Ltd. or Fisons.

2.2 SYNTHESIS OF D-PENICILLAMINE L-CYSTEINE DISULPHIDE

The mixed penicillamine-cysteine disulphide (PSSC) was prepared as described by Crawhall et al. (1964)[231]. 1.5 g of L-cysteine HCl and 330 mg D-penicillamine HCl were dissolved in ammonium hydroxide (4N, 20 ml). Ferric chloride (0.2 ml, 5% w/v in water) was added and air drawn through the solution for 18 h via a wash bottle also containing 4 N ammonium hydroxide. The

precipitated cystine and ferric hydroxide were removed by centrifugation and the supernatant was evaporated at 60°C using a rotary evaporator. The resulting residue was washed with water (2x25 ml) and the washings were applied to an ion-exchange chromatographic column (Zerolit 325 in the H^+ form; 12 x 250 mm). The column was eluted with water (200 ml) followed by ammonium hydroxide (2 N, 250 ml). The ammonia was removed by evaporation in a rotary evaporator at 60°C, leaving a solid residue of the mixed disulphide (approximately 200 mg) contaminated with 6.3% penicillamine disulphide and 7.7% cystine. The structure was confirmed by NMR and infra-red spectroscopy.

2.3 HUMAN STUDIES

All human studies (patients and volunteers) received approval from the Bath Area Health District Research Ethics Committee. All volunteers were medically examined and clinical-biochemical tests were carried out before and after the studies.

a) Patients

Patients, both male and female, aged 35 to 74 years, admitted to the Clinical Pharmacology Unit (CPU) of the Royal United Hospital (RUH), Bath, with self-administered overdose of paracetamol (P) or aspirin (ASA) were studied. On admission, gastric lavage was performed on each patient in the Casualty Department. Patients were then transferred to CPU where they received glycine oral fluid or urinary alkalinisation treatment for ASA overdose, or methionine or parvolex (N-acetylcysteine) treatment for P overdose. All urine passed by the patients was collected whenever possible until they left the unit. Urine volume and pH were measured on the ward. Concentrations of aspirin

metabolites (i.e. SA, SUA and GA) or paracetamol metabolites (i.e. P, PG and PS) present in each sample were determined by colorimetric assay, HPLC and NMR as described in sections 2.7, 2.8 and 2.9 respectively.

For the D-penicillamine (PSH) study, samples were obtained from RA patients who visited the out-patient clinic of the Royal National Hospital for Rheumatic Diseases, Bath. Blood samples were withdrawn by venepuncture as requested by the Consultant. Plasma samples not required for routine laboratory analysis were pooled for the determination of penicillamine metabolites (e.g. PSH, PSSP, PSSC and MPSH) by NMR as described in section 2.10.1.3.

b) Volunteers

Three healthy male volunteers, aged 29, 42 and 43 years who were participating in a clinical study on acemetacin (ACE) in the Royal United Hospital, Bath, were studied. 90 mg of ACE were taken orally twice daily, for eight days. Urine samples were collected two hourly for six hours after the first and the last doses for ACE and indomethacin (IN) determination by NMR as described in section 2.9.

2.4 ANIMAL EXPERIMENTS

The experimental animals used were male Wistar Albino rats (Bath University strain) weighing between 240-280 g. The rats injected with phenobarbitone (PB) were housed individually, and other experimental rats were housed in groups of one to three rats per cage (NKP cage). Food pellets (Labsure diet, CRM) and water were allowed ad libertum and a constant light-dark cycle was maintained (12 h light 7.00 to 19.00, 12 h dark 19.00 to 7.00) at an ambient temperature of 24 ^{o}C.

Induction of Rat Liver Enzymes

Phenobarbitone (PB, 101.8 mg ml^{-1} sodium salt in isotonic saline) was administered by intraperitoneal injection using the following regime:

- day 1: 50.9 mg kg^{-1} body weight (b.w.)
- days 2 and 3: 101.8 mg kg^{-1} b.w.

3-methylcholanthrene (3MC, 18 mg ml^{-1} in corn-oil) was administered by intraperitoneal injection, once only at a dose of 40 mg kg^{-1} b.w.

Control rats for PB received intraperitoneal injections of 0.5 ml kg^{-1} b.w. of saline on the first day and 1.0 ml kg^{-1} b.w. of saline on the second and third days. Control rats for 3MC received a single injection of 2.2 ml kg^{-1} b.w. of corn-oil. After the last doses of PB or saline and 24 h after the 3MC or corn-oil injections, food was withheld from the animals for 24 h before they were killed by cervical dislocation. The livers were immediately removed and 10,000 g liver fractions and microsomes prepared as described in section 2.6.

2.5 STORAGE OF BIOLOGICAL SAMPLES

All urine and plasma samples were stored at -20°C prior to analysis. Urine was stored in approximatley 4 x 10 ml aliquots where possible. Plasma was stored in EDTA tubes. Prepared microsomes were similarly stored at -20°C.

2.6 RAT LIVER PREPARATIONS AND THEIR PROTEIN AND CYTOCHROME ASSAYS

Rat Liver Preparations

The procedure used was a modification of various published methods[232,233]. Once the livers had been removed from the control, PB or 3MC treated rats, the following preparations were immediately carried out at $0^{o}C$.

Each liver was weighed and homogenates were prepared using 2 volumes of isotonic 1.15% KCl-0.01 M Na^{+}/K^{+} phosphate buffer, pH 7.4, by homogenizing the minced liver with a teflon and ground-glass homogenizer for about 1 minute. To remove cell nuclei and mitochondria, the homogenates were centrifuged at 10,000 g for 15 min at $4^{o}C$ in a MSE High Speed Model 18 centrifuge. The supernatant fraction, hence called 10,000 g liver fraction, was taken for (a) protein determination (see below), (b) in-vitro metabolism (see section 2.10.2) and (c) microsomal preparation.

Microsomes were obtained after ultracentrifugation of the liver supernatant fraction at 100,000 g for 1 h at $4^{o}C$ in a Beckman Model L8-70M centrifuge. The microsomal pellets obtained were suspended in fresh 20% glycerol-0.01 M Na^{+}/K^{+} phosphate buffer, pH 7.4, so that the entire yield of microsomes from each rat was suspended in a final volume of 10 ml. This suspension was re-homogenised using a ground-glass hand driven homogenizer to obtain a uniform suspension and deep frozen at $-20^{o}C$, until required for cytochrome P-450/P-448 and other cytochrome determinations.

Protein Assay of 10,000 g Rat Liver Fractions

The method for measuring protein concentration of the 10,000 g liver fraction was based upon the colorimetric determination of Lowry *et al.* (1951)[36]. The coloured complex formed between the alkaline copper-phenol reagent and the tyrosine and tryptophan residues of the protein was proportional to the protein concentration.

To 0.4 ml of diluted 10,000 g liver fraction sample containing 20 to 100 μg of protein (200x dilution of 10,000 g liver fraction described in section 2.6a with saline) was added 4.0 ml alkaline copper solution (50 ml of 2% Na_2CO_3 in 0.10 N NaOH and 1 ml of 0.5% $CuSO_4.5H_2O$ in 1% NaK tartrate). The solution was mixed well and allowed to stand for 10 min at room temperature. 0.4 ml diluted Folin-Ciocalteau reagent (1 volume of the reagent plus 2 volumes of distilled water) was added rapidly, mixed and allowed to stand for 30 min at room temperature in the dark. The absorbance was read at 650 nm against the reagent blank using a Perkin-Elmer model 550S uv-vis spectrophotometer. The protein concentrations were calculated from a calibration curve prepared using purified bovine serum albumin as a standard. The calibration curve was linear over the concentration ranges 50-400 μg/ml in saline. A typical calibration curve is given in fig. 2.6.1.

Assay for microsomal cytochromes

The measurement of microsomal cytochrome P-450 (or P-448 and P-420) and cytochrome b_5 was based on the method of Omura and Sato (1964)[234] using the carbon monoxide (CO)-difference spectra. Since cytochrome P-420 is considered to represent degraded cytochrome P-450/P-448, the cytochrome P-420 concentration was added to the

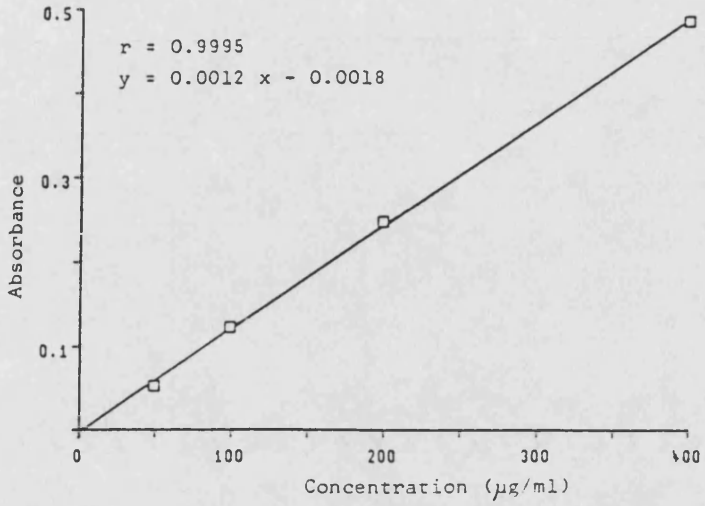


Fig. 2.6.1 Calibration curve for protein in rat liver supernatant fraction

observed cytochrome P-450/P-448 level to arrive at the total concentration of cytochrome P-450/P-448. The peak or shoulder at 420 nm was attributed to small amounts of contaminating haemoglobin as identified by the carbon monoxyhaemoglobin minus the oxyhaemoglobin spectrum.

a. Cytochromes P-450/P-448

The microsomal suspension was diluted with 20% glycerol- 0.01 M Na^+/K^+ phosphate buffer to give a protein concentration of approximately 2 mg/ml and placed in two cells, a reference and a sample cell. A baseline was recorded and the sample cell was subsequently gassed with carbon monoxide (CO) and its absorbance spectra (maximum about 420 nm) was scanned. This is referred to as the "haemoglobin trace". A few mg of sodium dithionite was then added to both cells, the carbon monoxide gassing of the sample cell repeated, and the absorbance spectrum re-scanned. This is referred to as the cytochrome

"P-450/P-420 or P-448/P-420 trace" (maximum about 450 nm or 448 nm and 420 nm).

The difference between absorbance at 420 nm on the latter trace and the haemoglobin trace, was added to the difference between absorbance at 450 nm or 448 nm and 480 nm on the P-450/P-420 or P-448/P-420 trace. The concentration of cytochrome P-450/P-448 was calculated from the above absorbance, assuming an extinction coefficient of 91 cm^{-1} $mmol^{-1}$ for the reduced hemoprotein-CO complex.

b. Cytochrome b_5

The above diluted microsomal suspension was placed in two matched cells. A baseline was recorded and then to the test cell was added a few mg sodium dithionite and both cells were re-scanned. The concentration of cytochrome b_5 was calculated from the absorbance difference between the maximum (about 428 nm) and 500 nm, assuming an extinction coefficient of 185 cm^{-1} $mmol^{-1}$ for the reduced haemoprotein-CO complex.

2.7 INSTRUMENTATION

2.7.1 ULTRAVIOLET-VISIBLE SPECTROPHOTOMETRY (SPEC)

Ultraviolet-visible absorption spectra were recorded with a microcomputer-controlled scanning double beam spectrophotometer (Perkin-Elmer model 550S; Perkin-Elmer & Co. GMBH, Uberlingen), with a model 561 recorder utilizing a matched pair of 1 cm path length silica cells (scan speed 60 nm/min, ordinate scale 0.0-3.0A.

2.7.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The HPLC system was assembled from commercially available components. The mobile phase was pumped to the column at constant flow using a Laboratory Data Control (LDC) Constametric III Metering Pump which provides a pulseless flow using dual-reciprocating pistons. The samples were introduced into the system using a Rheodyne injection valve fitted with a constant volume, 20 µl loop (Rheodyne 7205, Rheodyne Ltd., California, USA), and onto an analytical column (15 cm x 4.5 mm i.d.) packed with a reverse phase micro particle silica (Hypersil ODS 5 m, Shandon Product Ltd). In the case of aspirin analysis, a pre-column (5 cm x 4.5 mm i.d.) packed with identical material was placed in line immediately before the analytical column. The column end fitting comprised a low dead volume Swagelok connection with a $1/_{16}$ inch outline which was connected to the detector by microbore Teflon tubing (0.15 mm i.d.). Low dead volume $1/_{16}$ inch stainless steel tubing (0.15 mm i.d.) was used to connect the injection valve to the top of the column. All other connections were made of stainless steel tubing and standard Swagelok fittings (HETP Ltd., Macclesfield). Eluted compounds were detected using an LDC variable UV detector (Spectromonitor III). Chromatograms were recorded using a JJ Model CR550 potentiometric chart recorder (JJ Lloyd Instruments Ltd., Southampton).

All equipment was operated at ambient temperature throughout. Mobile phase was filtered through a 0.45 µm membrane filter (Millipore UK Ltd., London) and subsequently degassed by purging with helium for 5 min immediately prior to use.

2.7.3 PROTON NUCLEAR MAGNETIC RESONANCE (^{1}H NMR) SPECTROSCOPY

400 MHz ^{1}H spectra were obtained with a Bruker WH400 or a JEOL GX400 spectrometer and 270 MHz ^{1}H spectra with a JEOL GX270 spectrometer at ambient probe temperature (20 $\pm$ 1°C). Spectra were accumulated using 16K data points, a pulse delay of 5s to allow proton magnetization to recover by T_1 relaxation, and 32, 48 or 64 free induction decays (FIDs) either by single pulses or Carr-Purcell-Meiboom-Gill (CPMG) sequence $90^{\circ}(x)-(\tau-180^{\circ}(y)-\tau)_n-$ acqusition, τ = 60 ms. Accumulations were begun less than 2 min after introduction of the sample into the probe. The sample tube spinning speed was approximately 20 Hz, and the total spectral accumulation times for 48 FIDs were very short (5-10 min). The signal due to water was suppressed by homogated secondary irradiation. FIDs were zero-filled to 32 K data points before Fourier transformation, and either an exponential function corresponding to a 0.1 Hz line broadening or where indicated a Gaussian function for resolution enhancement was applied.

2.8 QUANTITATIVE ANALYSIS BY UV-VIS SPECTROPHOTOMETRY (SPEC)

2.8.1 ASPIRIN METABOLITES

Salicylic acid (SA), salicyluric acid (SUA), salicyl phenolic glucuronide (SPG) and salicyl acyl glucuronide (SAG) were assayed in human urine according to the method of Brodie et al. (1944)[235] with modifications by Trinder (1954)[34] and that for gentisic acid (GA) according to the method of Smith (1951)[35].

Assay Procedures

For the SA assay, 0.2 ml - 1.0 ml of urine samples (in duplicate) were extracted by adding 5 ml of carbon tetrachloride (CCl_4) and 0.2 ml of 6N HCl solution. The mixture was Whirli-mixed

for 1 min and then centrifuged at 3500 rpm for 5 min. The aqueous phase was discarded and 5 ml of 1% ferric nitrate was added to the organic phase. The mixture was mixed and centrifuged again as above. The absorbance of the aqueous phase was then read at 530 nm using a spectrophotometer. SA concentration is represented as 'A'.

For the SUA assay, the procedure was as for SA using methylene chloride (CH_3Cl) for extraction instead of CCl_4.

For the GA assay, 0.5 ml of urine samples (in duplicate) were extracted by adding 5 ml ether and 0.2 ml of 6N HCl. After 1 min Whirli-mixing and centrifugation at 3500 rpm for 5 min, the organic phase was placed in a clean tube and 5 ml of 5% $NaHCO_3$ was added. The mixture was mixed and cenrtrifuged as above and the ether phase was discarded. Finally, 6 ml of 6N HCl and 0.5 ml of Folin-Ciocalteau's reagent was added to the aqueous phase which was mixed and read in the spectrophotometer at 670 nm.

For the SPG assay, 0.1 ml-0.5 ml of urine samples (in duplicate) were adjusted to pH 5.0 with 1.0 ml acetate buffer and incubated with 0.3 ml of β-glucuronidase (5000 units/ml) at 37°C for 20 h. The samples were then reassayed for SA giving concentration (B) which represented the amount of SA+SPG. SPG concentration was taken to be (B-A).

For the SAG assay, 0.1 ml-0.5 ml of urine samples (in duplicate) were incubated with 1 ml of 2 N NaOH at 37°C for 20 h. At the end of the incubation period, the mixture was neutralized to pH 7.0 with 2N HCl and then reassayed for SA giving concentration (C) which represented the amount of SA+SAG. SAG concentration was taken to be (C-A).

Quantification

Quantification of aspirin metabolites in urine samples were calculated from the calibration curves prepared using standard SA, SUA or GA spiked in control human urine. Since SA is sparingly soluble in CH_3Cl and SUA is in CCl_4, concentrations of SA and SUA were achieved each by using two pairs calibration curves, i.e. SA and SUA extracted in CCl_4, and SA and SUA extracted in CH_3Cl. Calibration curves (absorbance vs. concentration) were constructed and were linear over the concentration ranges of 431-1725 μg/ml (SA extracted with CCl_4 or CH_3Cl), 500-2000 μg/ml (SUA extracted with CH_3Cl or CCl_4) and 100-300 μg/ml (GA). The coefficient of variation at 1725 μg/ml was 4% for SA extracted with either CCl_4 or CH_3Cl. At 431 μg/ml, the coefficients of variation were 6% for SA extracted with CCl_4 and 7% for SA extracted with CH_3Cl. The coefficients of variation at 2000 μg/ml were 3% for SUA extracted with CH_3Cl and 4% for SUA extracted with CCl_4. At 500 μg/ml, the coefficients of variation were 4% for SUA extracted with CH_3Cl and 9% for SUA extracted with CCl_4. At 300 μg/ml, the coefficient of variation was 2% for GA and at 100 μg/ml GA the value was 9%. Typical spectroscopic calibration curves are given in fig. 2.8.1, fig. 2.8.2 and fig. 2.8.3 for SA, SUA and GA respectively.

The metabolite concentrations were calculated using the following equation from the linear least-squares regression method:

$$y = mx+c \qquad (2.8.1)$$

where y is the observed absorbance value of metabolite, x is the unknown concentration to be determined and m and c are the regression coefficients calculated from the calibration curve. Accordingly, SA and SUA concentrations were determined from the following formulae which are derived from the two simultaneous

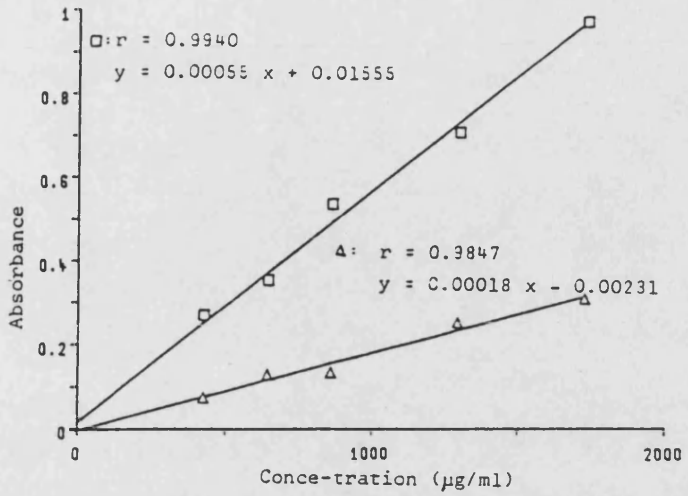


Fig. 2.8.1 Calibration curves for salicylic acid (SA) extracted with carbon tetra chloride ( ) and methylene chloride ( ) measured by spectrophotometry

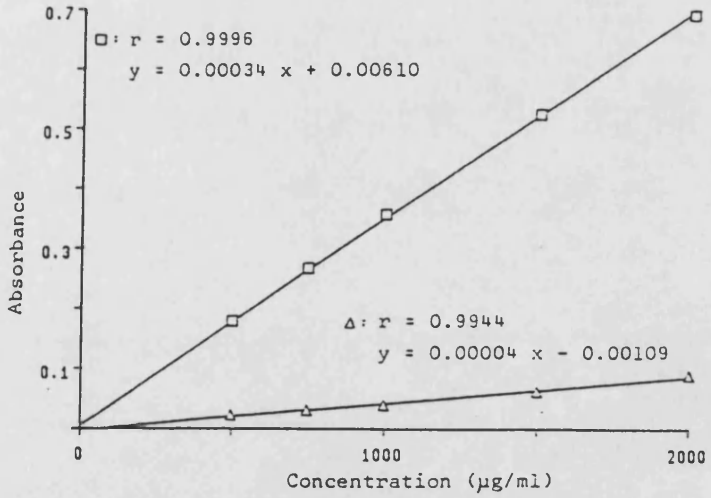


Fig. 2.8.2 Calibration curves for salicyluric acid (SUA) extracted with methylene chloride ( ) and carbon tetra chloride ( ) measured by spectrophotometry

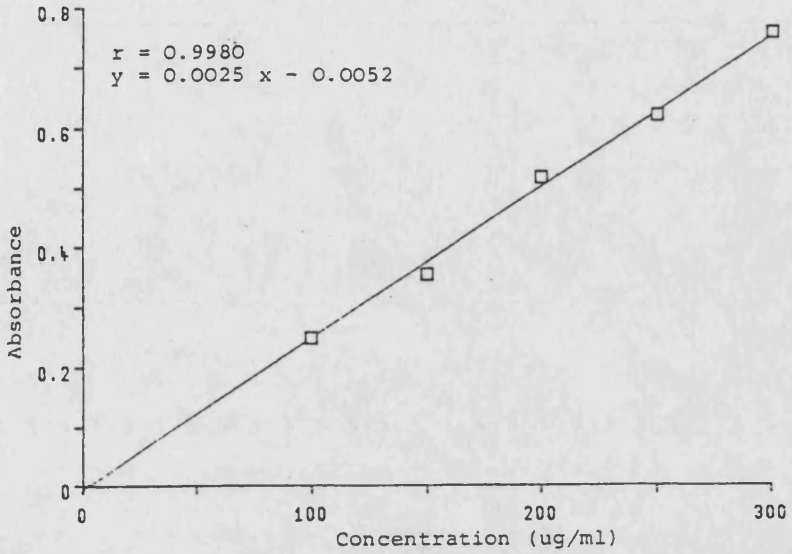


Fig. 2.8.3 Calibration curve for gentisic acid by SPEC

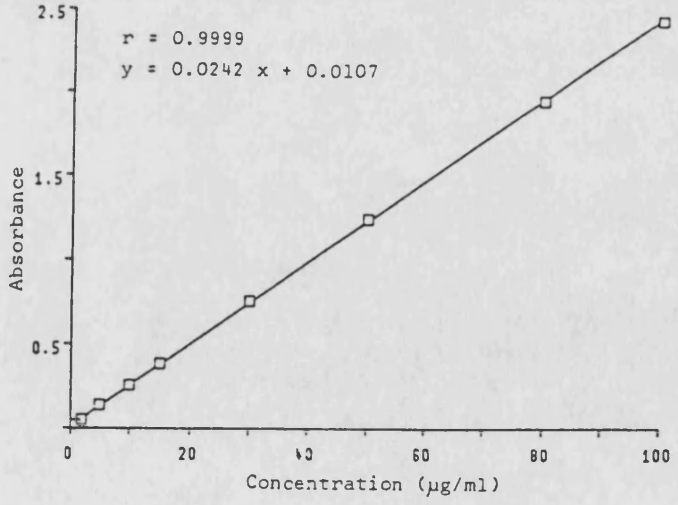


Fig. 2.8.4 Calibration curve for paracetamol by SPEC

equations, i.e. the total of SA and SUA extracted with CCl_4 and those with CH_3Cl.

$$y = m_1x_1+c_1 + m_2x_2+c_2$$
$$y' = m_1'x_1+c_1' + m_2'x_2+c_2' \qquad (2.8.2)$$

where y and y' are the observed absorbance values in CCl_4 and CH_3Cl respectively, x_1 and x_2 are the SA and SUA concentrations respectively, m_1, c_1 and m_2, c_2 are the regression coefficients calculated from the calibration curves of SA and SUA extracted with CCl_4 respectively, and m_1', c_1' and m_2', c_2' are the regression coefficients calculated from the calibration curves of SA and SUA extracted with CH_3Cl respectively.

2.8.2 PARACETAMOL AND ITS MAJOR METABOLITES

Paracetamol (P) was assayed according to Le Perdiel et al. (1968)[33] with modification from Chafetz et al. (1968)[236] for the determination of paracetamol (P), paracetamol glucuronide (PG) and paracetamol sulphate (PS) in human urine.

Assay Procedures

For the P assay, to 0.2 ml - 10 ml of urine samples (in triplicate) were added 0.4 ml of 6N HCl and 1.0 ml of sodium nitrite. After allowing the solution to stand for 15 min, 15 ml of 15% sulphonic acid was added to destroy excess nitrous acid. When nitrogen evolution had ceased, 3.0 ml of 10% NaOH was added and the solution was diluted to 10 ml (or 50 ml for the metabolite assay) with distilled water. The absorbance was then read at 430 nm using a spectrophotometer. Concentration of P was linearly related to absorbance. P concentration is represented as 'D'.

For the PG assay, 0.1 ml - 0.5 ml of urine samples (in triplicate) were adjusted to pH 5.0 with 1.0 ml acetate buffer and incubated with 0.3 ml of β-glucuronidase (5000 units/ml) at 37°C for 20 hours. The samples were then reassayed for P as described above giving concentration (E) which represented the amount of P+PG. PG concentration was taken to be (D-E).

For the PS assay, 0.1 ml - 0.5 ml of urine samples (in triplicate) were adjusted to pH 5.0 with 1.0 ml acetate buffer and incubated with 0.3 ml of β-glucuronidase (5000 units/ml) and 10 mg of sulphatase (19800 units/ml) at 37°C for 20 h. The samples were then reassayed for P as described above giving concentration (F) which represented the amount of P+PG+PS. PS concentration was taken to be (F-E).

Quantification

Quantification of P and its metabolites in urine samples were calculated from the calibration curve of P prepared using standard P spiked in control human urine. Calibration curve (absorbance vs. concentration) of P were constructed and were linear over the concentration ranges of 1-100 μg/ml. The coefficients of variation for P was 2% at 100 μg/ml and 9% at 1 μg/ml. A typical spectroscopic calibration curve for P is given in fig. 2.8.4. The P concentrations were calculated using the equation 2.8.1.

2.9 QUANTITATIVE ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

2.9.1 ASPIRIN METABOLITES

Salicylic acid (SA), salicyluric acid (SUA) and gentisic acid (GA) were assayed in human urine according to a modification of

method reported by Cham et al. (1980)[42], using o-anisic acid (AA) as the internal standard.

HPLC Conditions

Mobile phase : 25% acetonitrile
75% orthophosphoric acid (0.1%)
pH 2.5

Flow rate : 1.5 ml/min (operating pressure = 2000 psi)

Detection : 298 nm

Chart speed : 0.4 cm/min

Assay procedure

1 ml of urine samples (in duplicate) were added to 1 ml of internal standard (1.0 mg/ml AA in distilled water). 100 µl was injected into the column. Under these conditions, the retention times for GA, SUA or AA and SA in human urine were 2.6, 3.5, 4.1 and 7.6 min respectively. A typical chromatogram of GA (60 µg/ml), SUA (180 µg/ml), AA (250 µg/ml) and SA (120 µg/ml) spiked in control human urine is shown in fig. 2.9.1A. A chromatogram of a diluted urine sample from an aspirin overdose patient is shown in fig. 2.9.1B.

Quantification

Concentrations of aspirin metabolites in urine samples were calculated using the method of peak height (metabolite/internal standard) ratios from calibration curves prepared using standard SA, SUA, GA, and AA spiked in control human urine. Calibration curves (peak height ratio vs. concentration) were constructed and observed to be linear over the concentration ranges of 80-400 µg/ml (SA), 120-600 µg/ml (SUA) and 40-200 µg/ml (GA). The coefficients

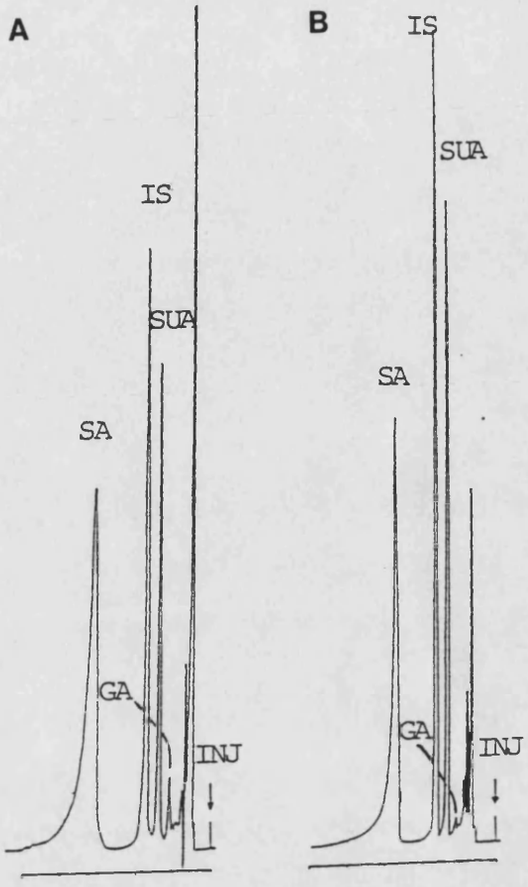


Fig. 2.9.1 Chromatograms of (A) GA (60 µg/ml), SUA (180 µg/ml), AA (250 µg/ml) and SA (120 µg/ml) spiked in control human urine and (B) urine sample from an aspirin overdose patient

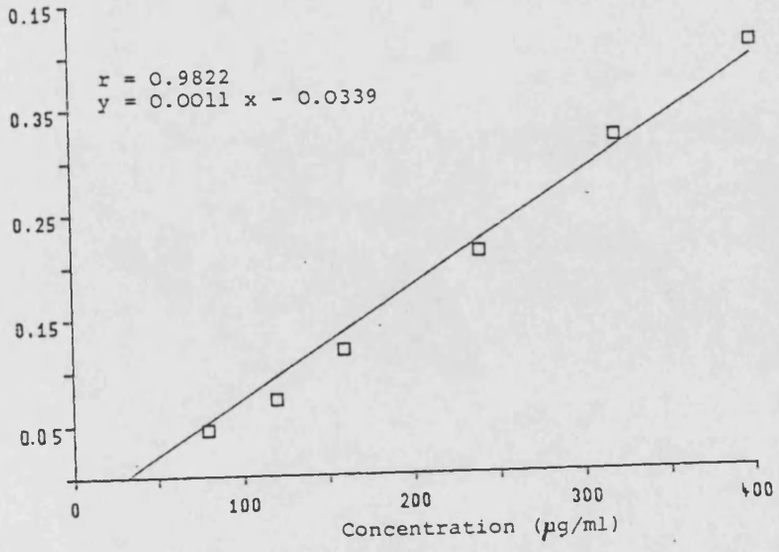


Fig. 2.9.2 Calibration curve for salicylic acid (SA) by HPLC

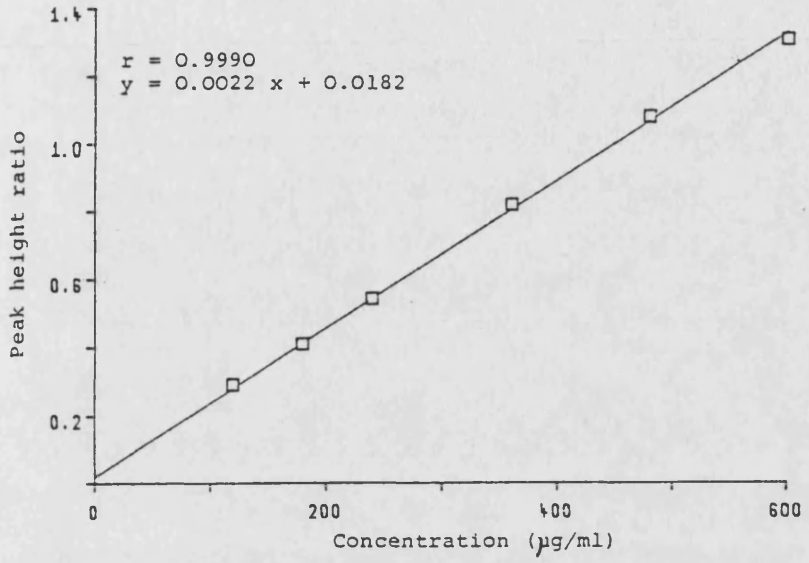


Fig. 2.9.2 Calibration curve for salicyluric acid by HPLC

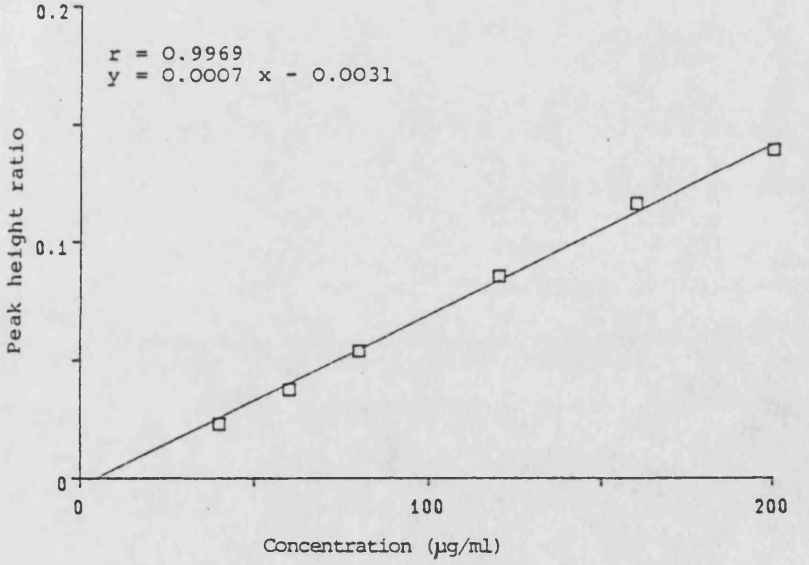


Fig. 2.9.3. Calibration curve for gentisic acid by HPLC

of variation for SA were 2% at 400 ug/ml and 7% at 80 ug/ml. At 600 ug/ml, the coefficient of variation was 1% for SUA and at 120 ug/ml SUA the value was 3%. The coefficients of variation for GA were 2% at 200 ug/ml and 5% at 40 ug/ml. Typical calibration curves by HPLC are given in fig. 2.9.2, fig. 2.9.3 and fig. 2.9.4 for SA, SUA and GA respectively.

The metabolite concentrations in patient samples were calculated using following equation from the linear least-squares regression method:

$$y = mx+c \qquad (2.9.1)$$

where y is the observed peak height ratio of metabolites to internal standard in urine, x is the unknown concentration to be determined and m and c are the regression coefficients calculated from the calibration curves.

2.9.2 PARACETAMOL AND ITS MAJOR METABOLITES

Paracetamol (P) was assayed according to a modification of method reported by Adriaenssens and Prescott (1978)[43], using gentisic acid (GA) as the internal standard, for the determination of paracetamol (P), paracetamol glucuronide (PG) and paracetamol sulphate (PS).

HPLC-Conditions:

Mobile phase : 94.9% 0.0 M KH_2PO_4
5.0% iso-propanol
0.1% formic acid
pH 3.0

Flow rate : 1.0 ml/min (operating pressure = 1000 psi)

Detection : 254 nm

Chart speed : 1.0 cm/min

Assay Procedures

For the P assay, 0.5 -1.0 ml of urine sample (in triplicate) was added to 1 ml of internal standard (10 mg/ml GA in distilled water) and made up to 10 ml with distilled water. 100 µl was injected onto the column.

For the PG and PS assays, the paracetamol metabolites were converted to free P as described for spectrophotometry (see section 2.8.2). The samples were then reassayed for P. Under these conditions, the retention times for P and GA in human urine were 3.5 and 5.1 minutes respectively. A typical chromatogram of P (0.2 mg ml^{-1}) and GA (1 mg ml^{-1}) spiked in control human urine is shown in fig. 2.9.5A. A chromatogram of a diluted urine sample from a paracetamol overdose patient is shown in fig. 2.9.5B.

Quantification

Concentrations of P and its metabolites in urine samples were calculated using the method of peak height (drug/internal standard) ratio. Calibration curves for P were prepared using standard P together with internal standard and GA spiked in control human urine. Calibration curves (peak height ratio vs. concentration) of P were constructed and were linear over the concentration range of 1-100 µg/ml. The coefficient of variation for P was 2% at 100 µg/ml and 4% at 1 µg/ml. A typical calibration curve for P by HPLC is given in fig. 2.9.6. P concentrations in urine samples were calculated using the equation 2.9.1 (see section 2.9.1).

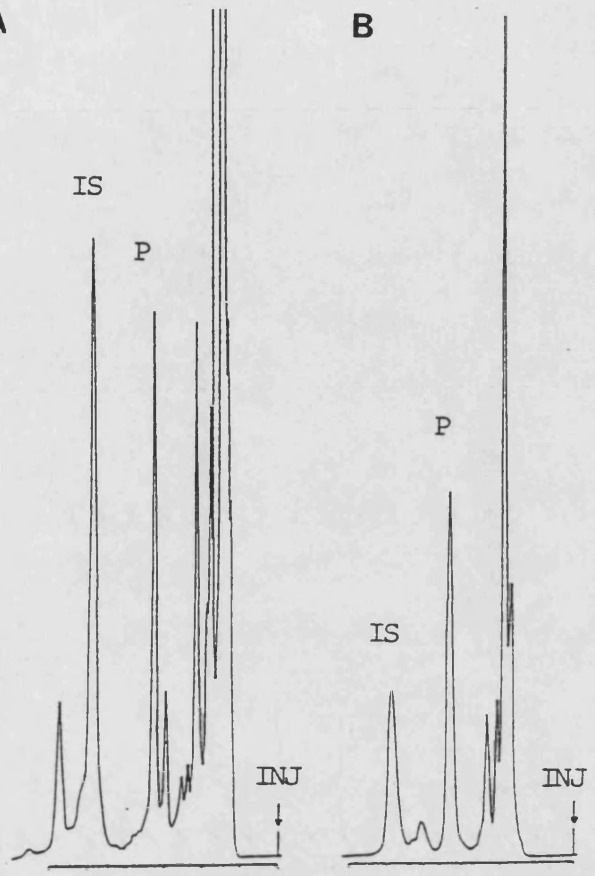


Fig. 2.9.5 Chromatograms of (A) P (0.2 mg/ml) and GA (1 mg/ml) spiked in control human urine and (B) urine sample from a paracetamol overdose patient

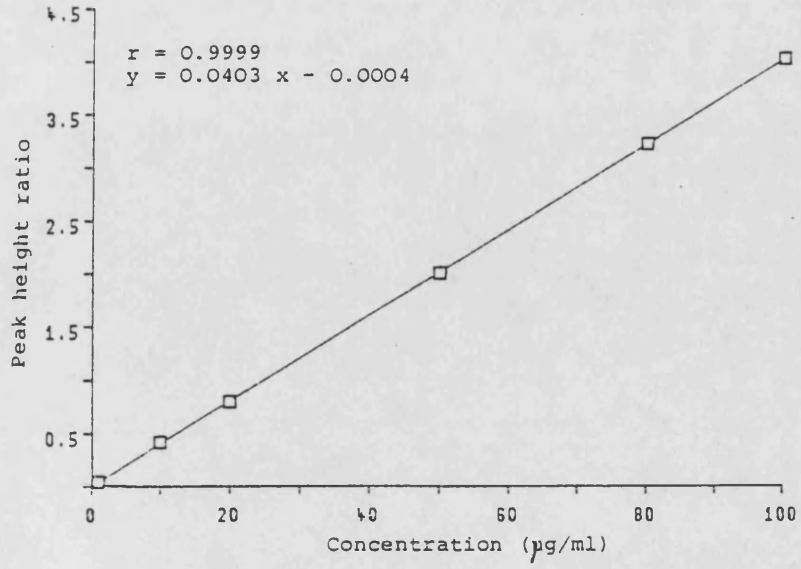


Fig. 2.9.6 Calibration curve for paracetamol (P) by HPLC

2.10 ANALYSIS BY PROTON NUCLEAR MAGNETIC RESONANCE (^{1}H NMR) SPECTROSCOPY

2.10.1 ANALYSIS OF DRUGS AND METABOLITES IN BODY FLUIDS

2.10.1.1 ASPIRIN , PARACETAMOL AND METABOLITES

Sample Preparation

Aliquots of urine (0.54 ml, intact or diluted with control urine) were placed in 5 mm NMR tubes and 0.06 ml of ^2H_2O as a field frequency lock containing internal standard (either valine 17.1 mM; or 2,2-dimethyl-2-silapentane-5-sulphonate DSS, 31.7 mM) were added.

^{1}H NMR Measurement:

The samples were run in a Bruker WH400 or JEOL GX400 spectrometer using the single pulse technique and homogated decoupling for water signal suppression (see section 2.7.3). Spectra were accumulated using 48 or 64 free induction decays (FIDs) and resolution enhancement was achieved by applying a Gaussian function to the FIDs.

Peak Assignments:

The assignments of resonances were confirmed by consideration of a combination of methods: standard additions, comparison with literature chemical shifts, analysis of coupling constants. Small amounts of some major components of urine were spiked into control human urine resulting in an increase of their relative magnitudes. Chemical shifts of paracetamol (P), its glucuronide (PG), sulphate (PS) and mercapturic (PMA) conjugates were recorded in 0.1 M deuterated phosphate buffer at pH* 5.6-7.3, referenced to DSS (δ= 0.0 ppm). Over this pH* range (pH read in ^2H_2O), there were no

substantial shifts (>0.02 ppm) in the resonance of paracetamol and its metabolites. ^{1}H NMR spectra of identical urine samples spiked with P, PG, PS and PNAC, salicylic acid (SA), salicyluric acid (SUA) and gentisic acid (GA) were recorded.The spectra were referenced to either the methyl signal of internal standard (δ = 1.0 ppm for valine, δ = 0.0 ppm for DSS) or the methylene signal of glycine (δ = 3.57 ppm) which do not shift in the normal pH range of urine (5-8)[94]. Samples were prepared and spectra were recorded using identical conditions for urine samples from paracetamol and aspirin overdose patients (see section 2.3a).

2.10.1.2 ACEMETACIN METABOLITE (INDOMETHACIN)

Sample preparation:

Urine samples were freeze-dried and redissolved inacetone-d_6-$^{2}H_2O$ (3:2 v/v) to concentrate them by 15 to 29 times. Lyophilized urine samples (0.54 ml) were placed in 5 mm NMR tubes and 0.06 ml of $^{2}H_2O$ containing internal standard DSS (4.2 mM) were added.

^{1}H NMR Measurement:

The samples were run in a JEOL GX270 spectrometer using the single pulse technique and homogated decoupling for water signal suppression (see section 2.7.3). Spectra were accumulated using 48 FIDs and resolution enhancement was achieved by applying a Gaussian function to the FIDs.

Peak Assignments:

The assignments of resonances were confirmed by consideration of a combination of standard additions, chemical shifts and coupling constants. Small amounts of some major components of urine were spiked into control human urine before being freeze-dried and redissolved in acetone-d_6-^2H_2O (3:2 v/v). ^1H NMR spectra of two identical lyophilized urine samples after spiking with acemetacin (ACE) or indomethacin (IN) were recorded. The spectra were referenced to either the methyl signal of DSS (δ = 0.0 ppm) or the methylene signal of glycine (δ = 3.57 ppm). Samples were prepared and spectra were recorded using identical conditions for urine samples from volunteers (see section 2.3b).

2.10.1.3 D-PENICILLAMINE AND ITS METABOLITES

Sample Preparation:

Pooled EDTA plasma samples were extracted with trichloroacetic acid (TCAA), freeze-dried, and redissolved in ^2H_2O to concentrate 40-80 times. Lyophilized plasma samples (0.54 ml) were placed in 5 mm NMR tubes and 0.06 ml of ^2H_2O containing internal standard DSS (8.5 mM) were added.

^1H NMR Measurement:

The samples were run in a JEOL GX400 spectrometer using single pulse technique and homogated decoupling for water signal suppression, then re-run using spin echo technique by CPMG (see section 2.7.3). Spectra were accumulated using 64 FIDs and resolution enhancement was achieved by applying a Gaussian function to the FIDs.

Peak Assignments:

The assignments of resonances were confirmed by consideration of a combination of standard additions, chemical shifts, coupling constants and phase characteristics in the spin echo spectra. Small amounts of some major components of plasma were spiked into the lyophilized normal EDTA plasma and the spectra re-run resulting in an increase of their relative magnitudes. To determine the analytical recovery, the ^{1}H NMR spectrum of freeze-dried normal EDTA plasma containing 0.3 mM of each penicillamine (PSH), penicillamine disulphide (PSSP), penicillamine cysteine (PSSC) and S-methyl penicillamine (MPSH) was recorded. The spectra were referenced to the methyl signal of DSS (δ= 0.0 ppm). Samples were prepared and spectra were recorded using identical conditions for pooled plasma samples from patients (see section 2.3a).

2.10.1.4 QUANTIFICATION OF PARACETAMOL AND ITS METABOLITES, AND ACEMETACIN METABOLITE (INDOMETHACIN)

Paracetamol (P) and its metabolites (PG and PS) were quantified by comparing the integrals (computer-integrated peak areas) of their acetyl methyl signals, expected to occur in regions devoid of resonances in normal urine, to those of the methyl signal of DSS (δ= 0 ppm) or valine (δ= 1.0 ppm) for a few samples. Indomethacin (IN) (acemetacin metabolite) was quantified by comparing the weights of paper traces of its methyl signal to those of the methyl signal of DSS. Only the single pulse spectra were used for quantification.

The following general equation is used for the calculation:

$$\frac{\text{Molarity of m}}{\text{Molarity of s}} = \frac{\text{Peak Integration of m}}{\text{Peak Integration of s}}$$

$$\frac{C_m MW_s}{C_s MW_m} = \frac{I_m N_s}{I_s N_m}$$

$$C_m = C_s \frac{I_m N_s MW_m}{I_s N_m MW_s} \qquad (2.10.2)$$

whereby C_m and C_s are the concentration of metabolite and standard respectively, I_m and I_s are the integrations or the weight of paper traces of metabolite and standard respectively, N_m and N_s are the numbers of their active nuclei and MW_m and MW_s are their molecular weights. To test the analytical reliability, calibration curves (integration ratio for P, PG and PS and weight of paper trace ratio for IN vs. concentration) were constructed and found to be linear over the concentration ranges 0.05-1.78 mg/ml (P), 0.40-2.00 mg/ml (PG), 0.22-1.80 mg/ml (PS) and 0.20-1.02 mg/ml (IN). The coefficients of variation for P were 2% at 1.78 mg/ml and 10% at 0.05 mg/ml. At 0.40 mg/ml the coefficient of variation was 2% for PG and at 2.00 mg/ml PG the value was 9%. The coefficients of variation for PS were 3% at 1.80 mg/ml and 11% at 0.22 mg/ml. The coefficients of variation for IN were 3% at 0.20 mg/ml and 11% at 1.02 mg/ml. Typical calibration curves for P, PG, PS and IN by ^1H NMR are given in fig. 2.10.1, 2.10.2, 2.10.3 and 2.10.4 respectively. Recoveries of P, PG, PS and IN in urine were 96.2±5.6%, 93.4±4.1%, 96.3±7.2% and 92.0±5.7% (mean+SD, n=4) respectively.

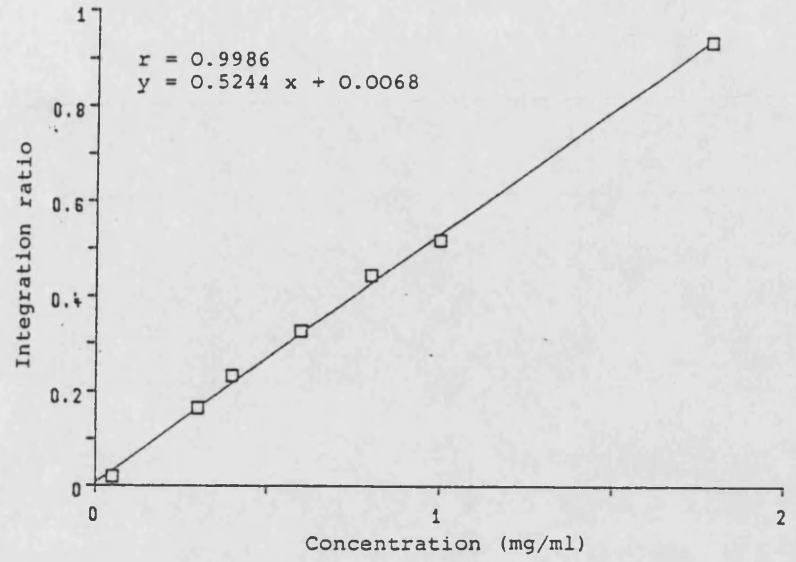


Fig. 2.10.1 Calibration curve for paracetamol by ^{1}H NMR

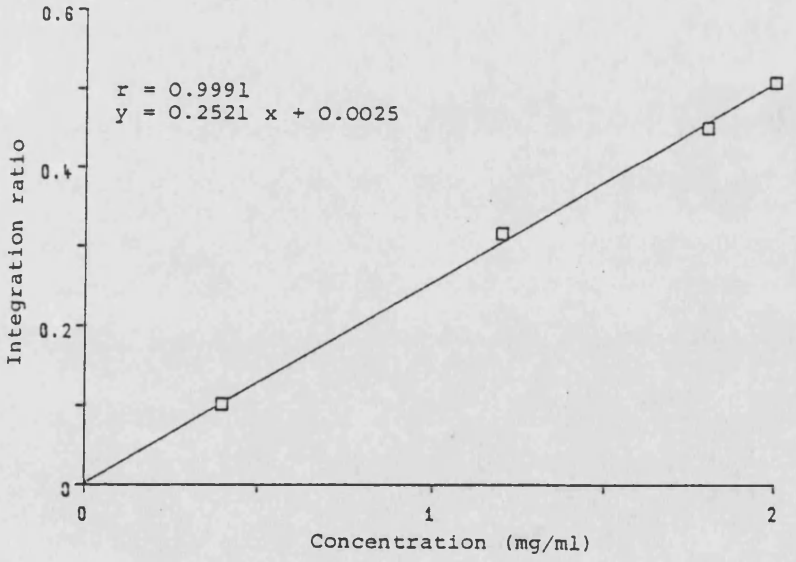


Fig. 2.10.2. Calibration curve for paracetamol glucuronide by ^{1}H NMR

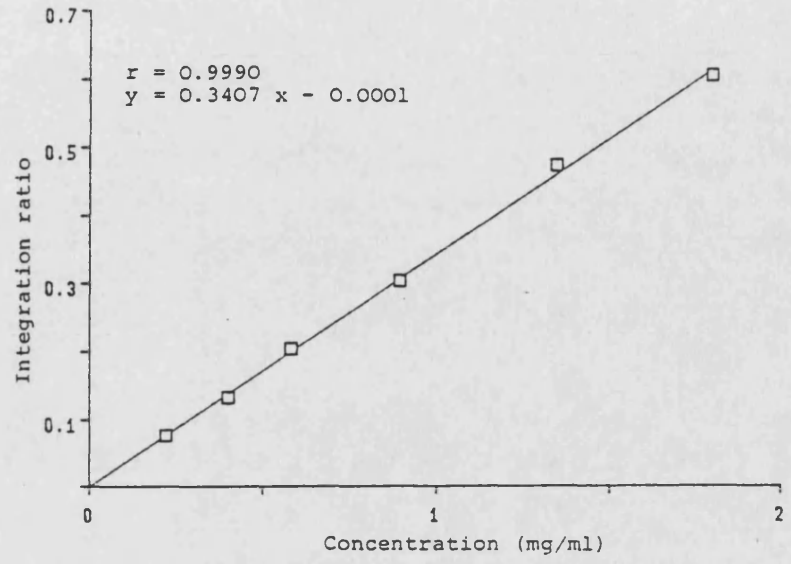


Fig. 2.10.3 Calibration curve for paracetamol sulphate by ^{1}H NMR

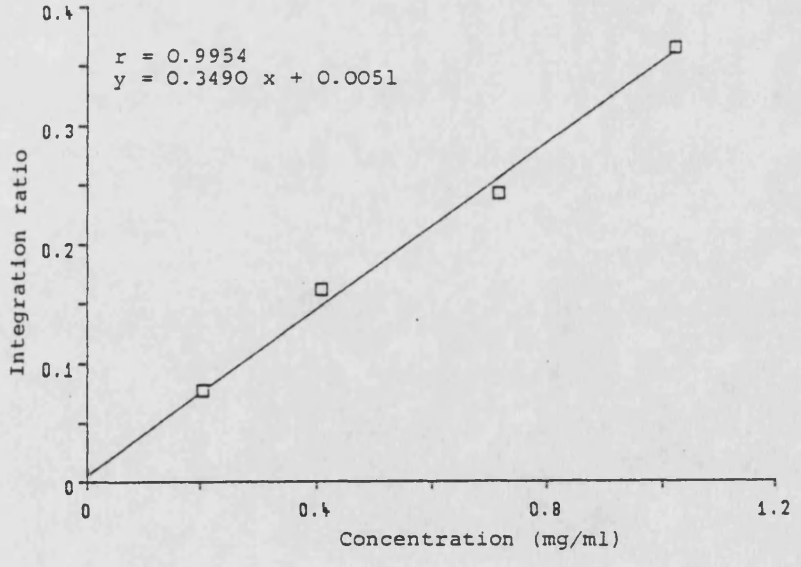


Fig. 2.10.4. Calibration curve for indomethacin by ^{1}H NMR

2.10.2 IN VITRO METABOLISM

Incubation Procedures:

The following solutions were added to a 10 ml flask in order:

1) 0.1 ml of aminopyrine (DMAP, 2 mM) or 0.1 ml daunorubicin (DA, 2 mM).
2) 0.1 ml of magnesium chloride ($MgCl_2$, 30 moles).
3) 0.2 ml of co-factor mixture containing nicotinamide adenine dinucleotide phosphate (NADP, 0.26 mole), glucose-6-phosphate (G6P, 6 moles) and nicotinamide (20 moles).
4) 0.6 ml of 10,000 g rat liver fraction containing enzyme equivalent to 30 mg protein.

Total volume 1.0 ml. All solutions were made up in 0.05 M Na^+/K^+ phosphate buffer pH 7.4. The reaction was carried out in a shaking water bath (120 cycles/min) at 37^o for 5,10,20,30 or 60 min in air. The blank mixtures (without added substrate) were incubated as controls using identical conditions. To stop the reaction, 0.1 ml of 55% trichloroacetic acid (TCAA) was added to the mixture. The mixture was then centrifuged at 4,000 g for 10 min and the spectra of the supernatant were recorded. The spectra of the above mixture were also recorded before and after protein precipitation (by addition of TCAA).

Sample Preparation:

Supernatants of deproteinated incubation mixtures (0.54 ml), hence called the acid extract of 10,000 g liver fraction, were placed in 5 mm NMR tubes and 0.06 ml of ^2H_2O as a frequency lock containing internal standard DSS (8.5 mM) were added.

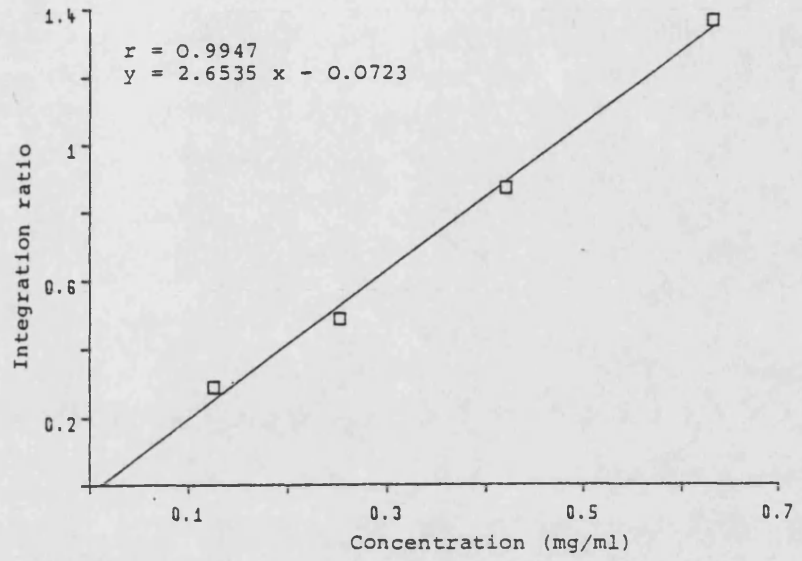


Fig. 2.10.6 Calibration curve for aminopyrine (DMAP) by ^{1}H NMR

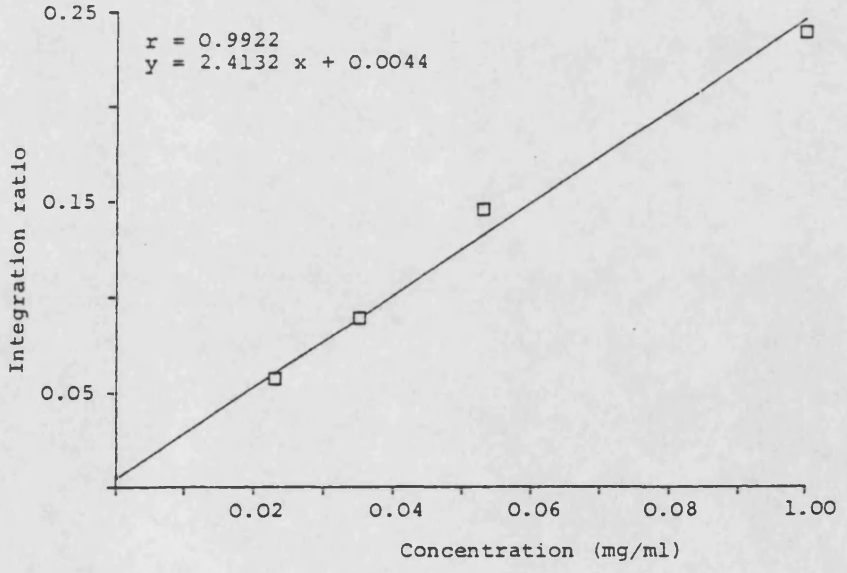


Fig. 2.10.7 Calibration curve for 4-aminoantipyrine (AP) by ^{1}H NMR

^{1}H NMR Measurement

The samples were run in a JEOL GX 270 using the single pulse technique and homogated decoupling for water signal suppression, then re-run using the spin echo technique by CPMG, collecting 32 FIDs in each case (see section 2.7.3).

Peak Assignment:

The assignments of resonances were confirmed by consideration of a combination of methods: standard additions, chemical shifts, and phase characteristics in the spin echo spectra. ^{1}H NMR spectra of three identical acid extracts of 10,000 g control liver fraction spiked with DMAP, 4-aminoantipyrine (AP) or DA were recorded and referenced to the methyl signal of DSS (δ=0.0 ppm).

Quantification of Aminopyrine and its metabolites

Aminopyrine (DMAP) and its metabolites were quantified by comparing the integrals of the peaks arising from methyl signals of DMAP, monomethyl-4-aminoantipyrine (MMAP) and AP which occurred in a region devoid of resonances in the control acid extract of 10,000g liver fraction, to the integral of methyl signal of DSS (δ= 0 ppm). The peaks in the single pulse spectra were quantified using equation 2.10.2 (see section 2.10.1.4). To get the optimum conditions for the 10,000 g liver fraction to metabolise DMAP, a series of identical mixtures containing various amount of 10,000 g liver fraction (i.e. enzymes equivalent to 10, 20, 30, 40 and 50 mg protein) were incubated for 30 min. The concentration of non-metabolised DMAP *vs.* mg protein is shown in fig. 2.10.5. The 10,000 g liver fraction enzymes equivalent to 30 mg protein was then chosen. To test the analytical reliability of DMAP and AP,

calibration curves (integration ratio vs concentration) were constructed and found to be linear over the concentration range 0.02-0.7 and 0.02-1.00 mg/ml respectively. Coefficient of variation at 0.02 mg/ml was 5% for DMAP and at 0.023 mg/ml AP, the value was 7%. At 0.7 mg/ml the coefficient of variation was 1% for DMAP and at 1.00 mg/ml AP, the value was 2%. Typical calibration curves for DMAP and AP in the acid extract of 10,000 g liver fraction are given in fig. 2.10.6 and 2.10.7. Recoveries of DMAP and AP were 97.3±2.8 % and 96.5±3.2% (mean±SD, n=4) respectively.

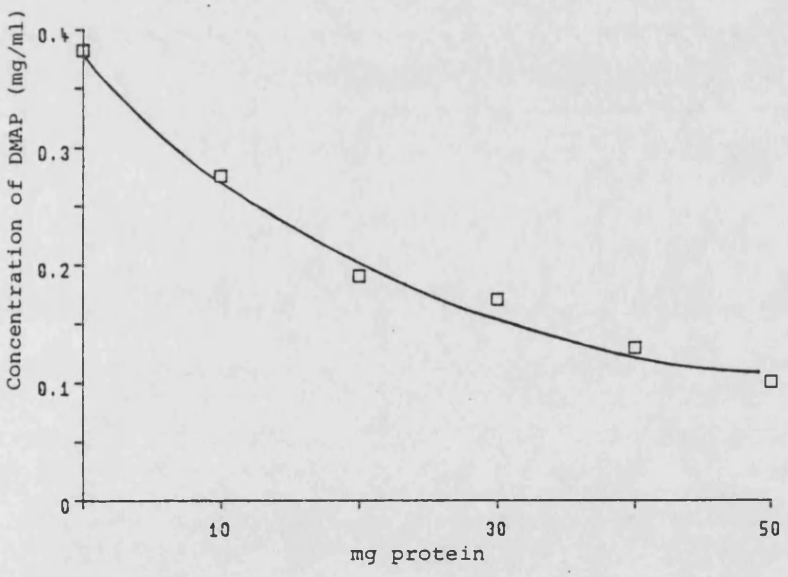


Fig. 2.10.5 Plot of non-metabolised DMAP concentrations vs mg protein in incubation

2.11 STATISTICAL ANALYSIS

Results are expressed where indicated as the mean ± standard deviation (SD). Further statistical analysis was performed using the computer statistical package, Instat. Statistical difference was determined using the Student's t-test (two-tailed, paired) and the degree of association or correlation assessed using Pearson's correlation coefficient (or product moment correlation coefficient). Statistical significance was taken at the 5% level.

CHAPTER THREE

RESULTS OF QUANTITATIVE STUDIES BY SPECTROPHOTOMETRY, HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND PROTON NUCLEAR MAGNETIC RESONANCE

Quantitative studies by proton nuclear magnetic resonance (^{1}H NMR) have been undertaken using four model drugs, i.e. aspirin (ASA), paracetamol (P), acemetacin (ACE) and D-penicillamine (PSH). In the case of aspirin and paracetamol, the ^{1}H NMR method was compared to the more conventional methods of visible spectrophotometry (SPEC) and high performance liquid chromatography (HPLC).

3.1 COMPARATIVE STUDIES OF ASPIRIN

In the present study, urinary excretion of aspirin (ASA) and its metabolites following intentional aspirin overdose has been analysed by visible spectrophotometry, high performance liquid chromatography and proton nuclear magnetic resonance. The following metabolites were analysed: salicylic acid (SA), salicyluric acid (SUA), gentisic acid (GA), salicyl phenolic glucuronide (SPG) and salicyl acyl glucuronide (SAG). The results obtained from the three methods were compared.

3.1.1 QUANTITATIVE ANALYSIS BY PROTON NUCLEAR MAGNETIC RESONANCE (^{1}H NMR) SPECTROSCOPY

Fig. 3.1.1 shows a typical 400 MHz spectrum of intact urine from a normal human subject after accumulation of 48 FID's obtained within 5 min. The metabolite resonances were identified by making standard additions of urinary components and considering their

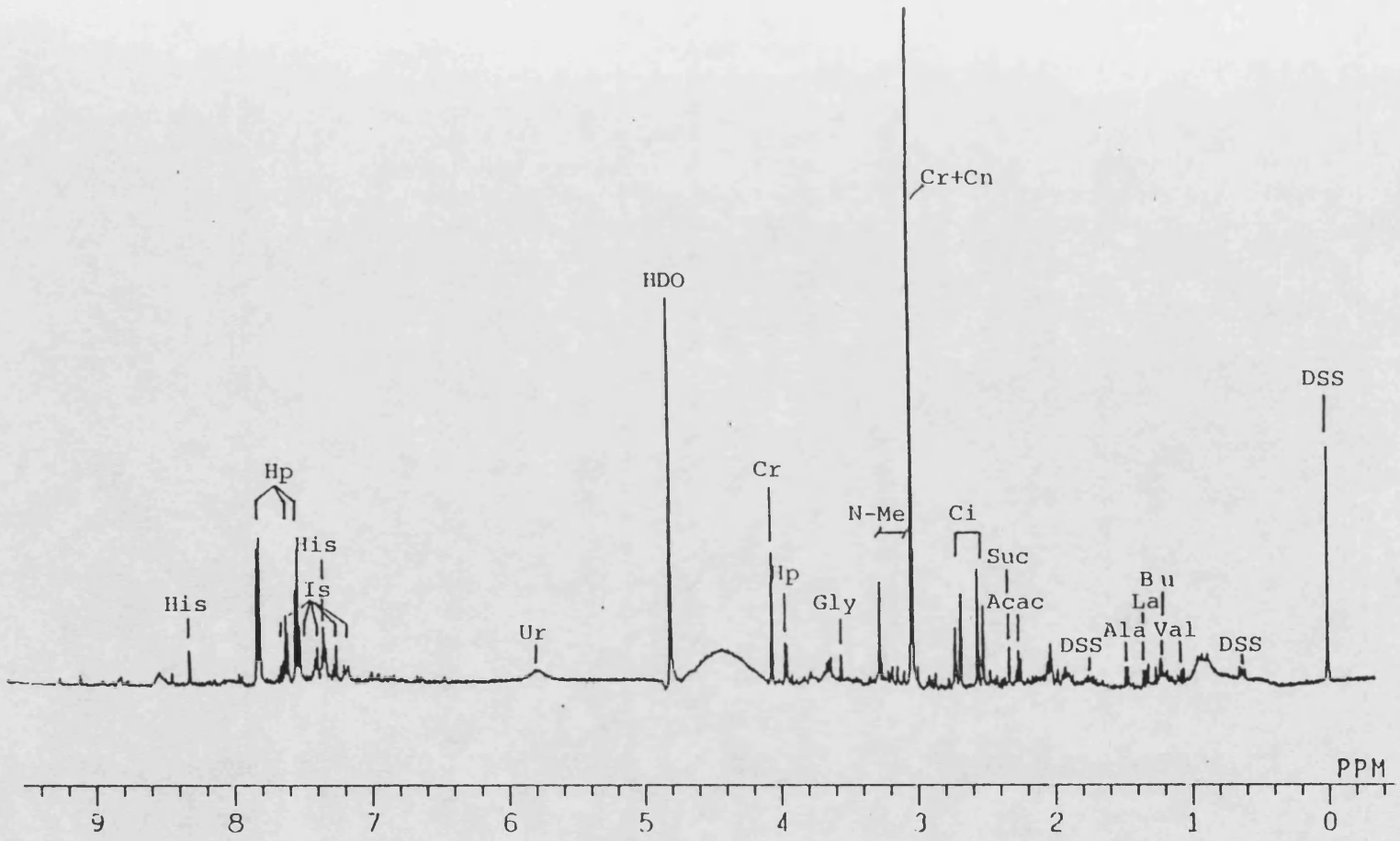
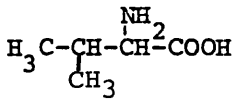
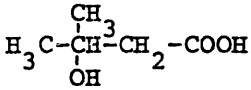
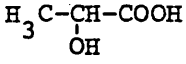
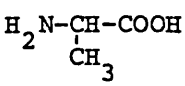
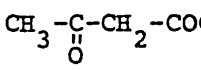
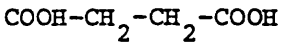
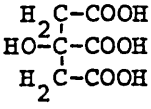
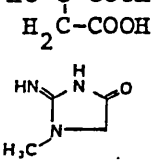
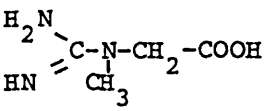
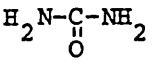
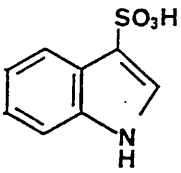
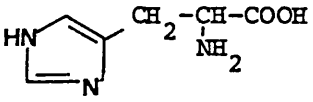
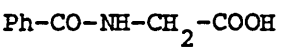


Fig. 3.1.1 400 MHz NMR spectrum of normal human urine containing 10% of $^{2}H_{2}O$. Accumulation of 48 FIDs at 24°C, using a 60° pulse, 5 s interval between pulses (PD), and 16 K data points. The water resonance was suppressed by homogated secondary irradiation. An exponential function corresponding to a 1-Hz line broadening was applied. Abbreviations for peak assignments are given in table 3.1.1. DSS = 2,2-dimethyl-2-silapentane-5-sulphonate as reference; N-Me = methyl resonances from choline head group.

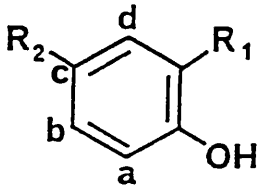
Table 3.1.1 Structures and 400 MHz ^{1}H NMR chemical shifts of some urinary endogenous metabolites.

Compound	Abbrev.	Structure	^{1}H Chemical Shift,[a] ppm
Valine	Val		1.07 (d)
β-Hydroxybutyrate	Bu		1.24 (d) 2.40 (ABX)
Lactate	La		1.34 (d)
L-Alanine	Ala		1.48 (d)
Acetoacetate	Acac		2.34 (s)
Succinate	Suc		2.38
Citrate	Ci		2.65 (AB)[b]
Creatinine	Cn		3.06 (s)[b]
Creatine	Cr		3.06 (s) 4.07 (s)
Glycine	Gly		3.57 (s)
Urea	Ur		5.80
Indoxyl sulphate	Is		7.19 (dd) 7.29 (dd) 7.40 (s) 7.49 (d) 7.69 (d)
Histidine	His		4.00 (t) 7.36 (s) 8.33 (s)
Hippurate	Hp		3.97 (d) 7.55 (t) 7.64 (t) 7.83 (d)

[a] As measured from fig. 3.1.2 for normal human urine at pH 5 (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, AB = 2nd - order spin system, ABX = AB part of ABX spin system). [b] Value depends on pH.

chemical shifts, peak intensities and characteristic coupling constants. Table 3.1.1 gives the structures and ^{1}H NMR chemical shifts of major endogenous metabolites in urine, referenced to the methyl signal of 2,2-dimethyl-2-silapentane-5-sulphonate (DSS). Aspirin metabolites, such as SA, SUA and GA, spiked into the human urine gave the chemical shift and coupling constant values shown in table 3.1.2. The ASA metabolites could be detected in the 400 MHz spectrum of a urine sample from a patient who had taken an overdose of ASA (fig. 3.1.2). However, their signals extensively overlapped with endogenous components and no suitable resonances could be quantified, therefore NMR analysis was not pursued further.

Table 3.1.2 Structures, 400 MHz ^{1}H NMR chemical shifts and coupling constants of aspirin metabolites in human urine



Name	Abbrev.	R Group	Chemical shift ppm	Coupling Constant J, Hz
Salicylic acid	SA	R_1=-COOH R_2=-H	H_a = 6.95 (d) H_b = 7.46 (t) H_c = 6.97 (t) H_d = 7.82 (d)	Jab = 7.5 Jac = 1.9
Salicyluric acid	SUA	R_1=-CONHCH$_2^e$COOH R_2=-H	H_a = 6.76 (d) H_b = 7.31 (t) H_c = 6.64 (t) H_d = 7.81 (d) H_e = 4.00 (s)	Jab = 7.5 Jac = 1.9
Gentisic acid	GA	R_1=-COOH R_2=-OH	H_a = 6.84 (d) H_b = 6.99 (dd) H_d = 7.30 (d)	Jab = 8.5 Jbc = 3.0

s = singlet, d = doublet, t = triplet, dd = doublet of doublets

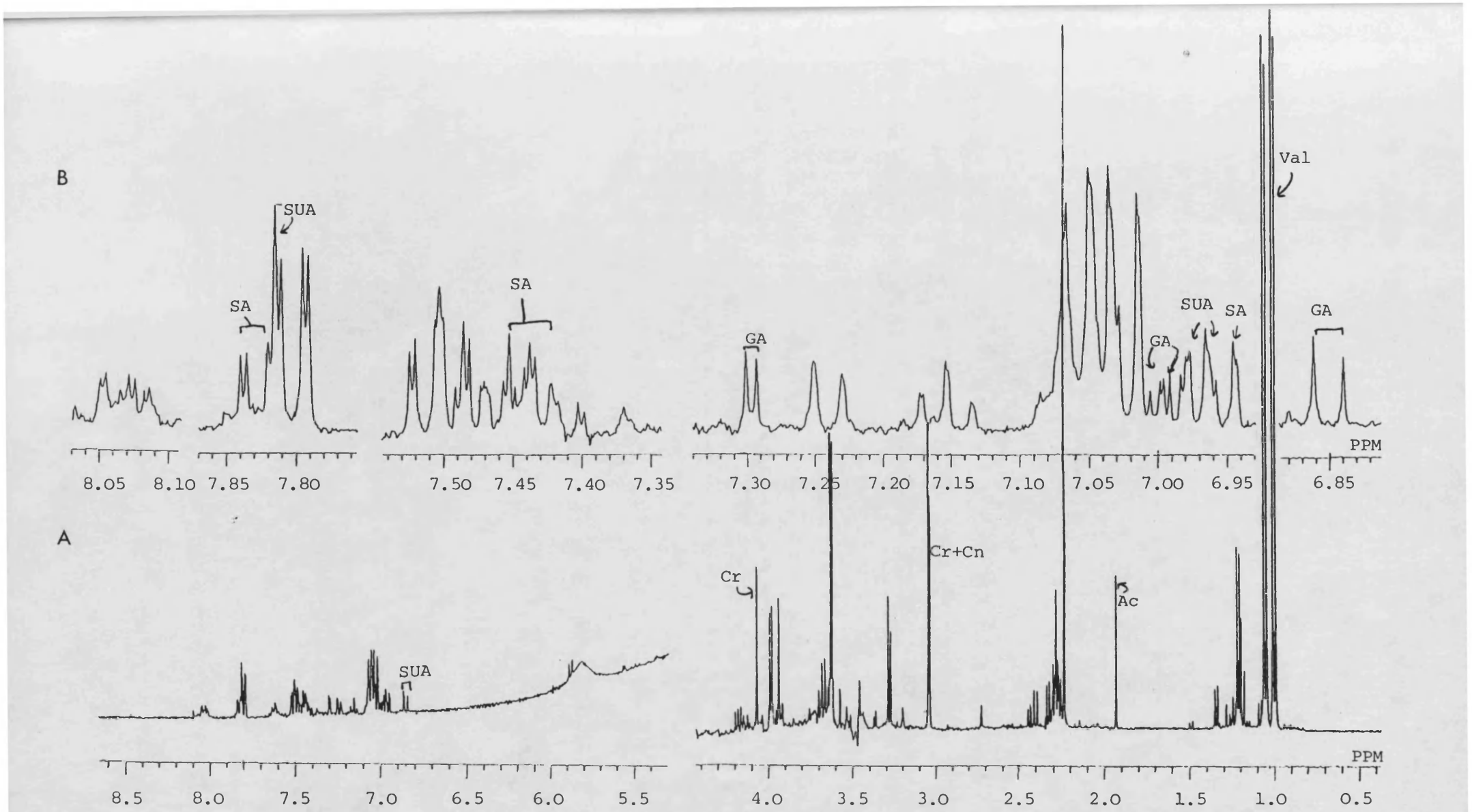


Fig. 3.1.2 400MHz NMR SPECTRUM OF A URINE SAMPLE FROM A PATIENT WHO HAD TAKEN ASPIRIN OVERDOSE. CONDITIONS FOR NMR ARE AS DESCRIBED IN FIG. 3.1.1 (B is an expansion of part of the spectrum given under 'A')

3.1.2 QUANTITATIVE ANALYSIS BY VISIBLE SPECTROPHOTOMETRY (SPEC)

Metabolite concentrations were calculated from calibration curves constructed for SA, SUA and GA (see section 2.8) based on the visible absorptions of each metabolite in SPEC. Measurements of SPG and SAG were obtained after their conversion to SA by enzyme hydrolysis (β-glucuronidase) and sodium hydroxide (NaOH) respectively, which when added to the urine samples resulted in the disappearance of SPG and SAG in HPLC chromatograms. Results are presented separately for each individual patient since the dose taken and the times of urine collections varied widely between the patients. Individual results and clinical details are given in tables 3.1.3-3.1.6 and are shown graphically in fig. 3.1.3 and 3.1.4. The results of concentration measurements of aspirin metabolites are shown in tables 3.1.7 - 3.1.9.

3.1.3 QUANTITATIVE ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATGRAPHY (HPLC)

ASA metabolites were identified on the basis of their retention times compared to standard metabolites, i.e. 2.6, 3.5, 4.1 and 7.6 min for GA, SUA, internal standard (O-anisic acid, AA) and SA respectively, under the conditions described in section 2.9. Individual results and clinical details are given in tables 3.1.3-3.1.6 and are shown graphically in fig. 3.1.3 and 3.1.4. The results of concentration measurements of aspirin metabolites are shown in tables 3.1.7 - 3.1.9.

Table 3.1.3 Clinical events and mg of urinary aspirin metabolites excreted from a patient (Al, male 21 yr) after aspirin overdose following treatment with glycine.

Time h	Event	Volume of urine (ml)	mg excreted of SA and its metabolites[a] HPLC SA	HPLC SUA	HPLC GA	SPEC SA	SPEC SUA	SPEC GA	SPEC SPG	SPEC SAG
18.00	Estimated time of 100 tablets of aspirin ingested									
18.00[b]	Admitted to Casualty Dept. Plasma salicylate 400 ug/ml									
19.00	Admitted to C.P.U. Dextrose administered (1000 ml)									
20.15	Glycine administered (8g)									
20.30	Plasma salicylate 360 ug/ml									
22.15	Glycine administered(4g)	700	230.9	246.8	122.1	210.6	279.2	172.3	98.4	33.1
22.30	Plasma salicylate 320 ug/ml									
23.00	Glycine administered (4g)	710	67.5	82.6	15.7	49.0	102.4	43.3	31.1	9.0
00.15	Glycine administered (4g)									
02.15	Glycine administered (4g)									
04.15	Dextrose administered (1000 ml) Glycine administered (4g)									
05.15	Glycine administered (4g)									
08.15	Glycine administered (4g)									
08.45		700	90.7	387.2	65.0	93.8	448.2	66.6	170.3	63.8
10.00		750	26.1	183.4	17.5	24.8	218.6	45.0	81.3	30.2
10.15	Glycine administered (4g)									
13.00		750	26.7	299.2	29.7	22.6	349.1	57.0	137.5	51.0
13.15	Glycine administered (4g)									
17.00		600	40.9	143.8	1.1	21.6	175.2	19.8	64.3	16.6
23.30		540	43.6	485.5	23.8	21.6	463.0	31.3	74.1	15.5
Total of individual metabolites (mg)			526.4	1828.5	274.9	444.0	2035.7	435.3	657.0	219.2
	(%)[c]		20.0	69.5	10.5	11.7	53.7	11.5	17.3	5.8
Total recovery (mg)[c]			2629.8			3791.2				

[a] Expressed as mg of SA equivalents.
[b] 24 h after the previous time.
[c] Based on total SA, SUA and GA in HPLC; and SA, SUA, GA, SPG and SAG in SPEC.

Table 3.1.4 Clinical events and mg of urinary aspirin metabolites excreted from a patient (A2, male 21 yr) after aspirin overdose following treatment with glycine.

Time h	Event	Volume of urine (ml)	mg excreted of SA and its metabolites[a] HPLC			SPEC				
			SA	SUA	GA	SA	SUA	GA	SPG	SAG
16.30	100 tablets of aspirin ingested									
17.45	Admitted to Casulaity Dept.									
18.00	Gastric wash-out									
21.00	Admitted to C.P.U.									
02.30	Plasma salicylate 410 ug/ml									
03.30	5% Dextrose administered (500 ml) Glycine administered (8g)									
03.45		850	254.0	298.7	NM[b]	170.2	374.6	25.0	110.5	32.3
22.30	Plasma salicylate 320 ug/ml									
05.30	Glycine administered (4g)									
07.15	Glycine administered (4g)	1650	837.5	250.6	42.7	794.8	243.7	83.2	21.4	107.2
07.30	Glycine administered (4g)									
08.30	Plasma salicylate 368 ug/ml									
09.30	Glycine administered (4g)									
11.15		750	116.6	127.3	NM	90.8	132.8	23.8	45.7	49.5
11.30	Glycine administered (4g)									
13.30	5% Dextrose administered (1000 ml) Glycine administered (4g)	870	133.0	217.5	20.0	98.8	261.6	68.2	146.2	59.2
15.30	Glycine administered (4g)									
16.30	Plasma salicylate 280 ug/ml	980	113.0	165.4	14.9	88.9	150.5	66.7	159.7	67.6
17.30	Glycine administered (4g)									
18.10		400	43.6	131.7	20.6	42.6	158.0	37.2	80.0	28.8
19.30	Glycine administered (4g)									
23.30		580	32.0	486.7	28.4	22.0	537.4	40.6	235.5	99.2
11.55		580	25.9	895.8	14.7	27.2	946.8	37.9	364.2	142.1
Total of individual metabolites (mg)			1555.6	2573.7	141.3	1335.3	2805.4	382.6	1163.2	585.9
(%)[c]			36.4	60.3	3.3	21.3	44.7	6.1	18.5	9.4
Total recovery (mg)[c]			4270.6			6272.4				

[a] Expressed as mg of SA equivalents.
[b] Not measure due to very low concentration.
[c] Based on total SA, SUA and GA in HPLC; and SA, SUA, GA, SPG and SAG in SPEC.

Table 3.1.5 Clinical events and mg of urinary aspirin metabolites excreted from a patient (A3, female 37 yr) after aspirin overdose following treatment with glycine.

Time h	Event	Volume of urine (ml)	mg excreted of SA and its metabolites[a] HPLC			SPEC				
			SA	SUA	GA	SA	SUA	GA	SPG	SAG
18.00	Estimated time overdose asprin ingested.									
07.45	Admitted to Casulaity Dept.									
08.00	Gastric wash-out									
10.00	Admitted to C.P.U. Plasma salicylate 500 ug/ml									
10.25	5% Dextrose administered (1000 ml)									
10.30	Glycine administered (8g)									
11.00		500	243.4	522.8	37.6	211.1	580.1	68.8	277.1	209.9
12.30	Glycine administered (4g)									
13.00		600	36.2	121.8	63.1	44.2	154.5	85.3	83.0	35.3
14.00	Plasma salicylate 320 ug/ml									
14.30	Glycine administered (4g)									
15.30		750	28.0	99.5	75.0	43.2	128.5	91.9	57.9	22.5
16.30	Glycine administered (4g)									
17.00		700	10.4	209.6	168.5	21.2	244.3	195.1	135.6	71.8
18.30	Glycine administered (4g)									
22.20	Glycine adminsitered (4g)									
22.30		200	6.1	155.1	73.9	10.6	191.9	73.5	88.5	48.2
24.10	Glycine administered (4g)									
10.00		600	26.9	632.2	129.3	38.4	712.5	142.7	279.5	125.9
Total of individual metabolites (mg)			351.0	1741.0	547.4	368.7	2011.8	657.3	921.6	513.6
	(%)[b]		13.3	66.0	20.7	8.2	45.0	14.7	20.6	11.5
Total recovery (mg)[b]			2639.4			4473.0				

[a] Expressed as mg of SA equivalents.
[b] Based on total SA, SUA and GA in HPLC; and SA, SUA, GA, SPG and SAG in SPEC.

Table 3.1.6 Clinical events and mg of urinary aspirin metabolites excreted from a patient (A4, male 45 yr) after aspirin overdose following treatment with glycine and bicarbonate.

Time h	Event	Volume of urine (ml)	mg excreted of SA and its metabolites[a] HPLC SA	HPLC SUA	HPLC GA	SPEC SA	SPEC SUA	SPEC GA	SPEC SPG	SPEC SAG
18.00	75 tablets of aspirin ingested									
18.43	Admitted to Casualty Dept.									
19.00	Gastric wash-out									
21.00	Admitted to C.P.U. Plasma salicylate 600 ug/ml									
22.20	5% Dextrose administered (1000 ml) Glycine administered (8g)									
00.20	5% Dextrose administered (500 ml) Glycine administered(4g)									
01.00	Plasma salicylate 660 ug/ml	750	1210.6	227.4	76.0	1100.0	230.9	92.7	96.8	ND[b]
02.30	5% Dextrose administered (1000 ml) Glycine administered (4g)									
03.00	1.26% Bicarbonate administered (500 ml)	550	44.1	107.1	43.5	447.3	108.3	48.7	1.3	ND
04.30	Glycine administered (4g) 1.26% Bicarbonate adminitered (500 ml)	875	225.7	27.5	NM[c]	195.1	57.7	40.9	14.1	ND
05.00		550	257.1	48.6	36.8	227.7	38.7	28.1	17.8	ND
06.30	5% Dextrose administered (1000 ml)									
08.30	Glycine administered (4g) Plasma salicylate 450 ug/ml									
10.00		2660	600.9	127.1	80.9	488.4	218.1	89.4	67.6	ND
10.30	Glycine administered (4g)									
12.35	Glycine administered (4g)									
12.45		1225	186.2	74.2	44.2	78.4	111.5	57.9	48.0	ND
14.35	Glycine administered (4g)									
17.00		1580	209.7	65.7	NM	154.0	104.3	88.5	96.5	27.3
19.10		720	78.5	73.4	29.8	58.8	93.5	39.9	46.5	8.3
06.00		1565	167.9	712.9	146.6	93.9	863.9	165.7	369.8	185.8
Total of individual metabolites (mg)			2980.7	1463.9	457.8	2843.6	1826.9	661.8	758.4	221.4
	(%)[d]		60.8	29.9	9.3	45.1	28.9	10.5	12.0	3.5
Total recovery (mg)[d]			4902.4			6312.1				

[a] Expressed as mg of SA equivalents.
[b] Not detected.
[c] Not measured due to very low concentration.
[d] Based on total SA, SUA and GA in HPLC; and SA, SUA, GA, SPG and SAG in VIS.

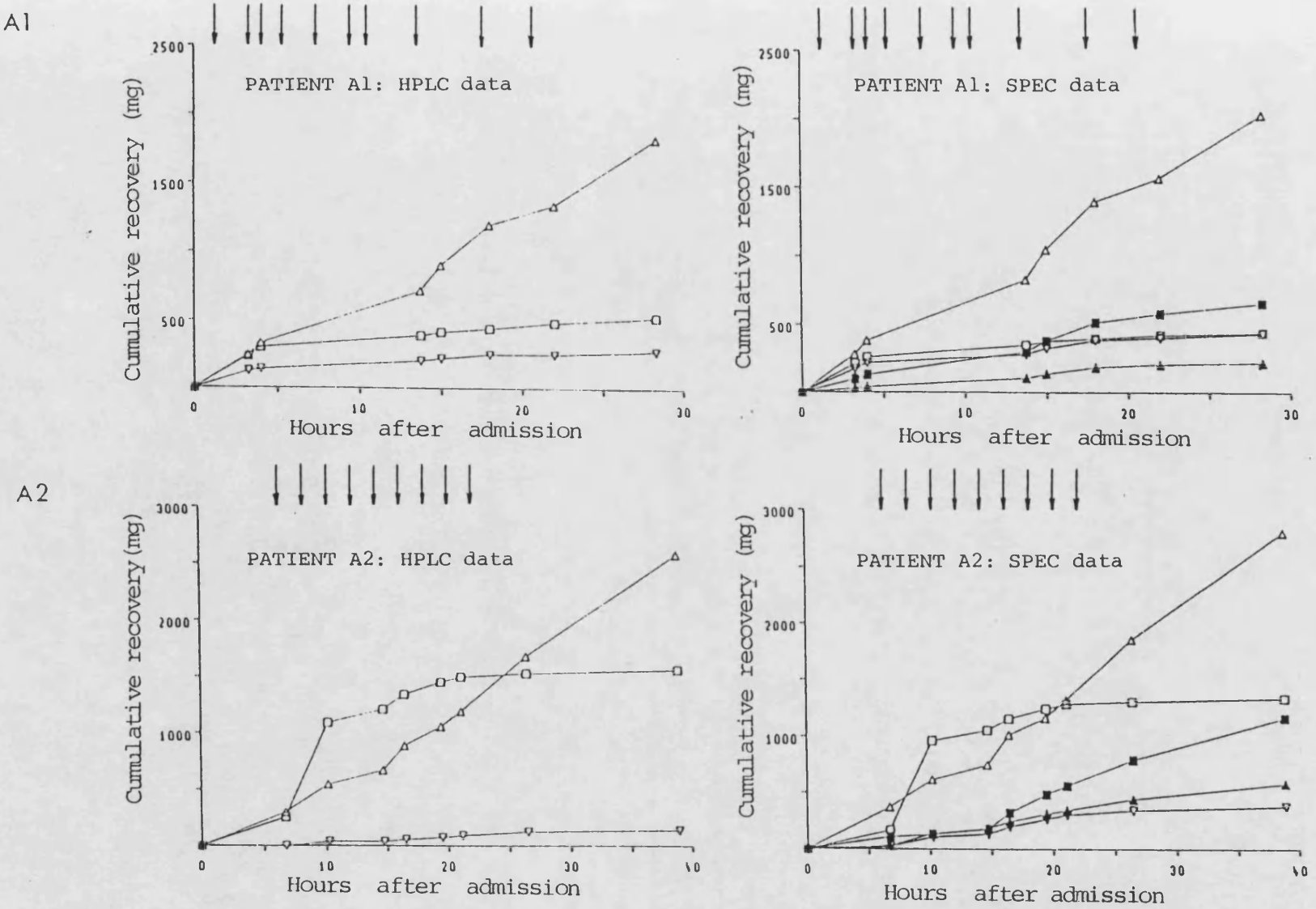


Fig. 3.1.3 Plots of cumulative recovery of aspirin metabolites as a function of time in the urine of two patients measured by HPLC and SPEC. Patient events are given in tables 3.1.3 and 3.1.4. Arrows indicate glycine administration. □ = salicylic acid; ▽ = gentisic acid; △ = salicyluric acid; ■ = salicyl phenolic glucuronide; ▲ = salicyl acyl glucuronide.

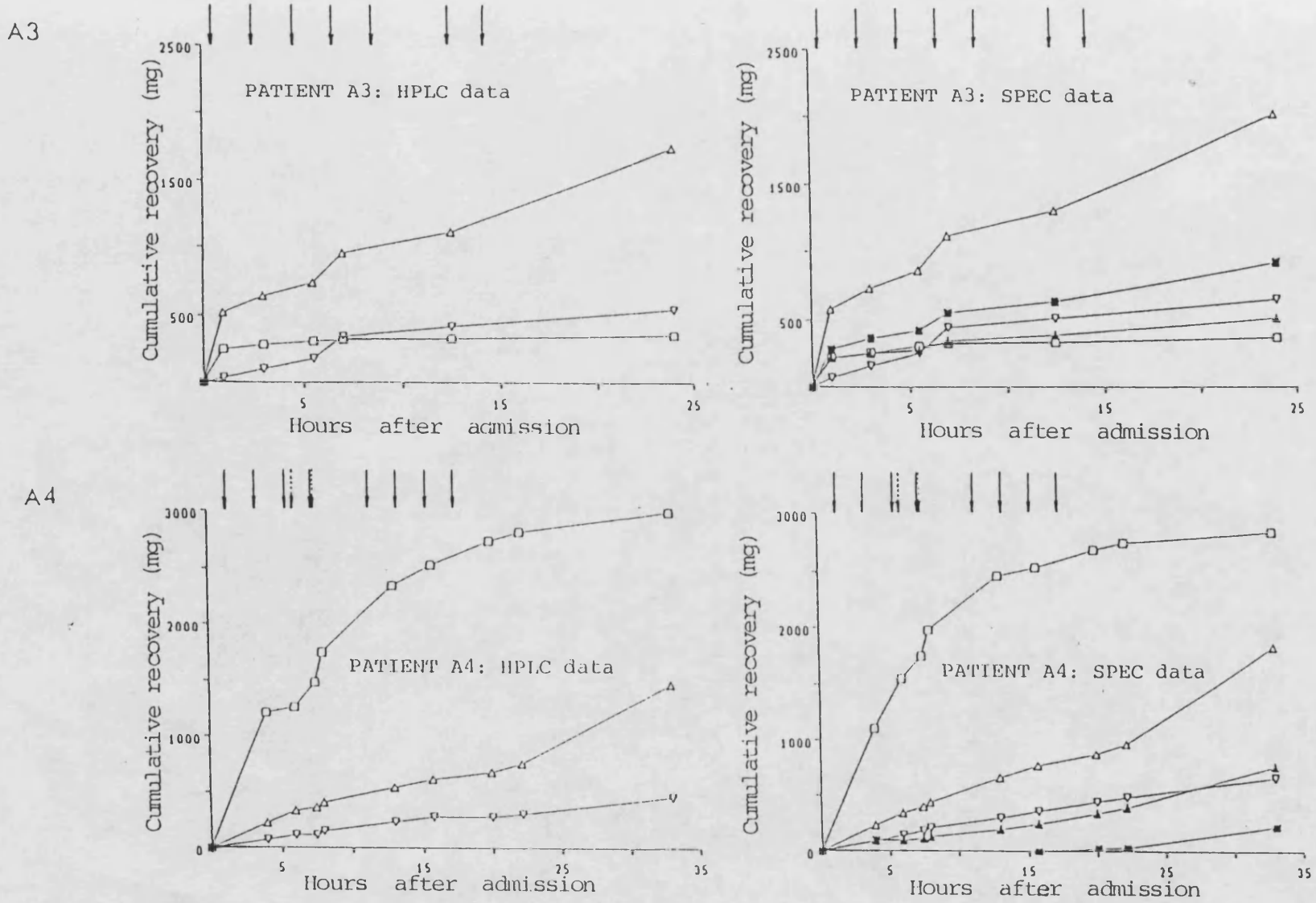


Fig. 3.1.4 Plots of cumulative recovery of aspirin metabolites as a function of time in the urine of two patients measured by HPLC and SPEC. Patient events are given in tables 3.1.5 and 3.1.6. Arrows indicate the administration of gycine ⟶ or bicarbonate ⇢. □ = salicylic acid; ▽ = gentisic acid; △ = salicyluric acid; ■ = salycil phenolic glucuronide; ▲ = salicyl acyl glucuronide.

Table 3.1.7 Quantitation of aspirin metabolite, salicylic acid (SA) in urine samples from aspirin overdose patients, based on HPLC and SPEC

Subject	Time of Collection[a], h.min	Concentration (mM) SUA HPLC	Concentration (mM) SUA SPEC	% Difference SPEC/HPLC
Al	0 - 3.15	2.56	2.89	112.9
male	3.15- 4.00	0.84	1.04	123.8
21 yr	4.00-13.45	4.00	4.63	115.7
	13.45-15.00	1.77	2.11	119.2
	15.00-18.00	2.89	3.37	116.6
	18.00-22.00	1.74	2.11	121.2
	22.00-28.30	6.51	6.21	95.4
Pearson's[b]			r= 0.990 (P<0.001)	
Student's[c]			t=-6.90 (P<0.002)	
A2	0 - 6.45	2.54	3.19	125.6
male	6.45-10.15	1.10	1.07	97.3
21 yr	10.15-14.45	1.23	1.28	104.1
	14.45-16.30	1.81	2.81	155.2
	16.30-19.30	1.22	1.11	91.0
	19.30-21.10	2.38	2.86	120.2
	21.10-26.30	6.07	6.71	110.5
	26.30-38.55	11.18	11.82	105.7
Pearson's			r= 0.998 (P<0.001)	
Student's			t= 3.00 (P<0.002)	
A3	0 - 1.00	6.88	7.64	111.0
female	1.00- 3.00	1.47	1.86	126.5
37 yr	3.00- 5.30	0.96	1.24	129.2
	5.30- 7.00	2.16	2.53	117.1
	7.00-12.30	5.61	6.94	123.7
	12.30-24.00	7.63	8.59	112.6
Pearson's			r= 0.997 (P<0.001)	
Student's			t= 4.11 (NS)	
A4	0 - 4.00	2.19	2.23	101.8
male	4.00- 6.00	1.41	1.43	101.4
45 yr	6.00- 7.30	0.22	0.48	218.2
	7.30- 8.00	0.64	0.51	79.7
	8.00-13.00	0.35	0.59	168.6
	13.00-15.45	0.44	0.66	150.0
	15.45-20.00	0.30	0.48	160.0
	20.00-22.10	0.74	0.94	127.0
	22.10-33.00	3.29	4.00	121.6
Pearson's			r= 0.985 (P<0.001)	
Student's			t=-2.47 (P<0.05)	

[a] Admission time to C.P.U. is taken as zero time
[b] HPLC versus SPEC data (mean value used)
[c] Paired t-test, comparisons with HPLC data
NS = not significant

Table 3.1.8 Quantitation of aspirin metabolite, salicyluric acid (SUA) in urine samples from aspirin overdose patients, based on HPLC and SPEC

Subject	Time of Colloection[a] h.min	Concentration (mM) SUA HPLC	SPEC		% Difference SPEC/HPLC
Al	0 - 3.15	2.39	2.18		191.2
male	3.15- 4.00	0.69	0.50		72.5
21 yr	4.00-13.45	0.94	0.97		103.2
	13.45-15.00	0.25	0.24		96.0
	15.00-18.00	0.26	0.22		84.6
	18.00-22.00	0.49	0.26		53.1
	22.00-28.30	0.59	0.29		49.1
Pearson's[b]			r= 0.986	(P<0.001)	
Student's[c]			t=-2.89	(P<0.05)	
A2	0 - 6.45	2.16	1.45		67.1
male	6.45-10.15	3.68	3.49		94.8
21 yr	10.15-14.45	1.12	0.88		78.6
	14.45-16.30	1.11	0.82		73.9
	16.30-19.30	0.83	0.66		79.5
	19.30-21.10	0.79	0.77		97.5
	21.10-26.30	0.40	0.27		67.5
	26.30-38.55	0.33	0.34		103.0
Pearson's			r= 0.981	(P<0.001)	
Student's			t=-2.72	(P<0.05)	
A3	0 - 1.00	3.20	2.78		86.9
female	1.00- 3.00	0.43	0.54		125.6
37 yr	3.00- 5.30	0.27	0.42		155.5
	5.30- 7.00	0.11	0.22		200.0
	7.00-12.30	0.22	0.38		172.7
	12.30-24.00	0.33	0.46		139.4
Pearson's			r= 0.999	(P<0.001)	
Student's			t= 0.44	(NS)	
A4	0 - 4.00	11.69	10.62		90.8
male	4.00- 6.00	5.84	5.89		100.8
45 yr	6.00- 7.30	1.87	1.61		86.1
	7.30- 8.00	3.38	3.00		88.7
	8.00-13.00	1.64	1.33		81.1
	13.00-15.45	1.10	0.46		41.8
	15.45-20.00	0.96	0.70		72.9
	20.00-22.10	0.79	0.59		62.0
	22.10-33.00	0.77	0.43		55.8
Pearson's			r= 0.992	(P<0.001)	
Student's			t=-3.60	(P<0.01)	

[a] Admission time to C.P.U. is taken as zero time
[b] HPLC versus SPEC data (mean value used)
[c] Paired t-test, comparisons with HPLC data
NS = not significant

Table 3.1.9 Quantitation of aspirin metabolite, gentisic acid (SA) in urine samples from aspirin overdose patients, based on HPLC and SPEC

Subject	Time of Collection[a], h.min	Concentration (mM) GA HPLC	GA SPEC	% Difference SPEC/HPLC
Al	0 - 3.15	1.26	1.78	141.3
male	3.15- 4.00	0.16	0.44	275.0
21 yr	4.00-13.45	0.67	0.69	103.0
	13.45-15.00	0.17	0.43	252.9
	15.00-18.00	0.29	0.55	189.6
	18.00-22.00	0.01	0.24	2400.0
	22.00-28.30	0.32	0.42	131.2
Pearson's[b]			r= 0.961 (P<0.01)	
Student's[c]			t= 3.97 (P<0.01)	
A2	0 - 6.45	NM	0.21	-
male	6.45-10.15	0.19	0.36	189.5
21 yr	10.15-14.45	NM	0.23	-
	14.45-16.30	0.17	0.56	329.4
	16.30-19.30	0.11	0.49	445.4
	19.30-21.10	0.37	0.67	181.1
	21.10-26.30	0.35	0.51	145.7
	26.30-38.55	0.18	0.47	261.1
Pearson's			r= 0.549 (NS)	
Student's			t= 6.70 (P<0.01)	
A3	0 - 1.00	0.49	0.90	183.7
female	1.00- 3.00	0.76	1.03	135.5
37 yr	3.00- 5.30	0.72	0.88	122.2
	5.30- 7.00	1.74	2.02	116.1
	7.00-12.30	2.67	2.66	99.6
	12.30-24.00	1.56	1.72	110.3
Pearson's			r= 0.992 (P<0.001)	
Student's			t= 3.59 (P<0.02)	
A4	0 - 4.00	0.73	0.90	123.3
male	4.00- 6.00	0.57	0.64	112.2
45 yr	6.00- 7.30	NM	0.34	-
	7.30- 8.00	0.48	0.50	104.2
	8.00-13.00	0.22	0.25	113.6
	13.00-15.45	0.26	0.34	130.8
	15.45-20.00	NM	0.40	-
	20.00-22.10	0.30	0.40	160.0
	22.10-33.00	0.68	0.77	113.2
Pearson's			r= 0.984 (P<0.001)	
Student's			t= 4.17 (P<0.01)	

[a] Admission time to C.P.U. is taken as zero time
[b] HPLC versus SPEC data (mean value used)
[c] Paired t-test, comparisons with HPLC data
NM = not measured, NS = not significant

3.1.4 COMPARISON OF RESULTS

Due to the lack of NMR results for ASA metabolites, only HPLC and SPEC data are discussed. The results comparing concentration measurements of ASA metabolites by HPLC and SPEC are shown in table 3.1.7-3.1.9. Statistical analysis of the data (table 3.1.7-3.1.9) by Pearson's correlation indicate that the concentrations of SA and SUA measured by SPEC were in good correlation ($r>0.98$, $P<0.001$) with those obtained on the same samples by HPLC. The concentrations of GA measured by SPEC were found to be in good correlation in three patients ($r>0.98$, $P<0.001$ for A3 and A4, $r>0.96$, $P<0.01$ for A1), but show no correlation in one patient ($r=0.5486$ for A2) with those by HPLC. Student's t-test for paired samples comparing SPEC results to those of HPLC showed only one result with no significant difference between the two methods (SA from A3). SA concentrations measured by SPEC in 3 patients (A1, A2 and A4) were found to be significantly lower ($P<0.05$, $P<0.05$ and $P<0.01$ respectively) than those by HPLC. SUA and GA concentrations measured by SPEC were significantly greater than those by HPLC in all patients, A1, A2, A3 and A4 (with $P<0.01$, $P<0.002$, $P<0.02$ and $P<0.01$ respectively for SA and $P<0.002$, $P<0.02$, $P<0.01$ and $P<0.05$ respectively for SUA).

It can be seen from tables 3.1.3-3.1.6 that the major metabolite recovered in patients receiving glycine treatment was SUA, measured by both HPLC or SPEC methods. However, SA appeared to be the major metabolite in a patient (A3) receiving glycine and bicarbonate treatment (table 3.1.6). The SPEC data shows approximately 45-54% SUA and 8-22% SA excretion in three patients (A1, A2 and A3) and 29% SUA and 45% SA excretion in one patient (A3). The ratios of SUA/SA, GA/SA and (SPG+SAG)/SA are also

Table 3.1.10 Urinary aspirin metabolites ratios to SA excreted in urine from patients taken aspirin overdose measured by HPLC and visible spectrophotometry

Patient	Time of Collection,[a] h.min	HPLC		SPEC		
		(SUA)/(SA)	(GA)/(SA)	(SUA)/(SA)	(GA)/(SA)	(SPG+SAG)/(SA)
A1	0-3.15	1.069	0.529	1.326	0.818	0.624
	3.15-4.00	1.224	0.233	2.090	0.884	0.818
	4.00-13.45	4.269	0.717	4.778	0.710	2.496
	13.45-15.00	7.027	0.670	8.814	1.814	4.496
	15.00-18.00	11.206	1.112	15.447	2.522	8.341
	18.00-22.00	3.516	0.027	8.111	0.917	3.745
	22.00-28.30	11.135	0.546	21.435	1.449	4.148
A2	0-6.45	1.176	-	2.201	0.147	0.839
	6.45-10.15	0.299	0.051	0.307	0.105	0.162
	10.15-14.45	1.092	-	1.462	0.262	1.048
	14.45-16.30	1.635	0.150	2.648	0.690	2.079
	16.30-19.30	1.464	0.132	1.693	0.750	2.557
	19.30-21.10	3.021	0.472	3.709	0.873	2.554
	21.10-26.30	15.209	0.887	24.43	1.845	15.214
	26.30-38.55	34.587	0.568	34.81	1.393	18.614
A3	0-1.00	2.148	0.154	2.748	0.326	2.307
	1.00-3.00	3.365	1.743	3.495	1.930	2.676
	3.00-5.30	3.554	2.679	2.974	2.127	1.861
	5.30-7.00	20.154	16.202	11.524	9.203	9.783
	7.00-12.30	25.426	12.115	18.104	6.934	12.896
	12.30-24.00	23.502	4.807	18.555	3.716	10.557
A4	0-4.00	0.188	0.063	0.210	0.084	0.088
	4.00-6.00	0.241	0.098	0.242	0.109	0.003
	6.00-7.30	0.122	-	0.296	0.210	0.072
	7.30-8.00	0.189	0.143	0.170	0.167	0.078
	8.00-13.00	0.211	0.135	0.447	0.183	0.138
	13.00-15.45	0.398	0.237	1.422	0.738	0.612
	15.45-20.00	0.313	-	0.677	0.575	0.627
	20.00-22.10	0.935	0.380	1.590	0.679	0.791
	22.10-33.00	4.246	0.873	9.200	1.765	3.938

calculated for each urine samples by HPLC and SPEC, as shown in table. 3.1.10.

3.2 COMPARATIVE STUDIES OF PARACETAMOL

Urinary excretion of paracetamol (P) and its metabolites from the patients after intentional P overdose has been analysed by SPEC, HPLC and ^{1}H NMR. The following metabolites were analysed: P, paracetamol glucuronide (PG) and paracetamol sulphate (PS) by the three methods, as well as paracetamol mercapturic acid (PMA) by ^{1}H NMR. The results obtained were compared.

3.2.1 QUANTITATIVE ANALYSIS BY PROTON NUCLEAR MAGNETIC RESONANCE (^{1}H NMR) SPECTROSCOPY

Numerous endogenous metabolites, including creatinine, citrate, hippurate, ketone bodies, and various amino acids, have been identified in 400 MHz ^{1}H NMR spectra of intact human urine (fig. 3.1.1, table 3.1.1). Fig. 3.2.1 and 3.2.2 show typical 400 MHz spectra for urine from a patient who had taken P overdose, referenced to the methyl resonance of valine (δ = 1 ppm). A reasonable signal-to-noise ratio was usually achieved after 64-128 accumulations, depending on the concentrations of the metabolites. Therefore, the spectrum of one sample could be obtained within 5-10 min using the conditions described in section 2.7.3 and 2.10.1. Table 3.2.1 shows the structure, chemical shifts and coupling constants of P and its metabolites based on standard additions and comparison with data of Bales *et al*[104]. Results are presented separately for each individual patient as the dose was unknown and the times of urine collections varied for each individual. Individual results and clinical details are given in

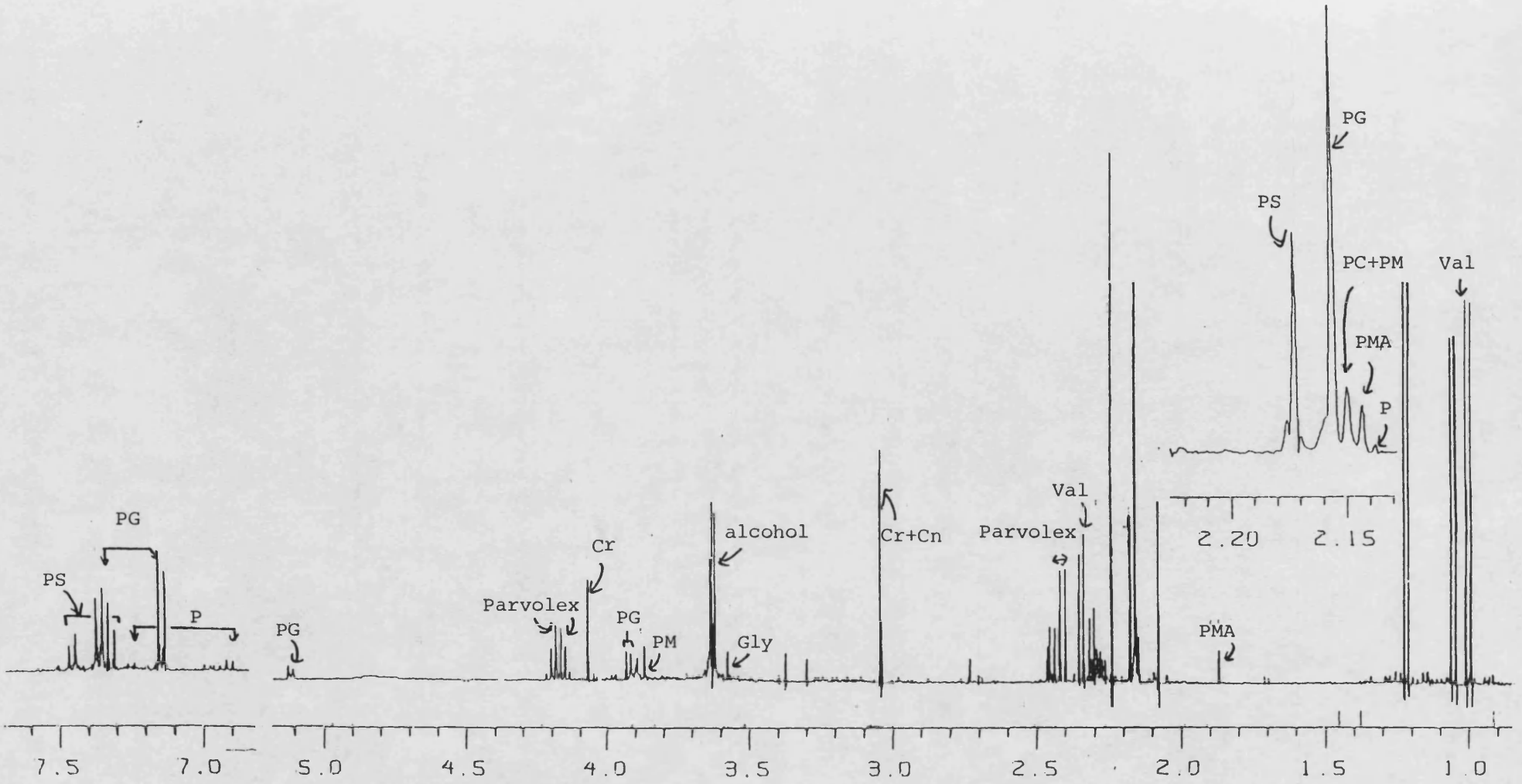


Fig. 3.2.1 400MHz NMR SPECTRUM OF A URINE SAMPLE FROM A PATIENT WHO HAD TAKEN PARACETAMOL OVERDOSE AND HAD BEEN TREATED WITH PARVOLEX (N-ACETYLCYSTEINE). CONDITIONS FOR NMR ARE AS DESCRIBED IN Fig. 3.1.1.

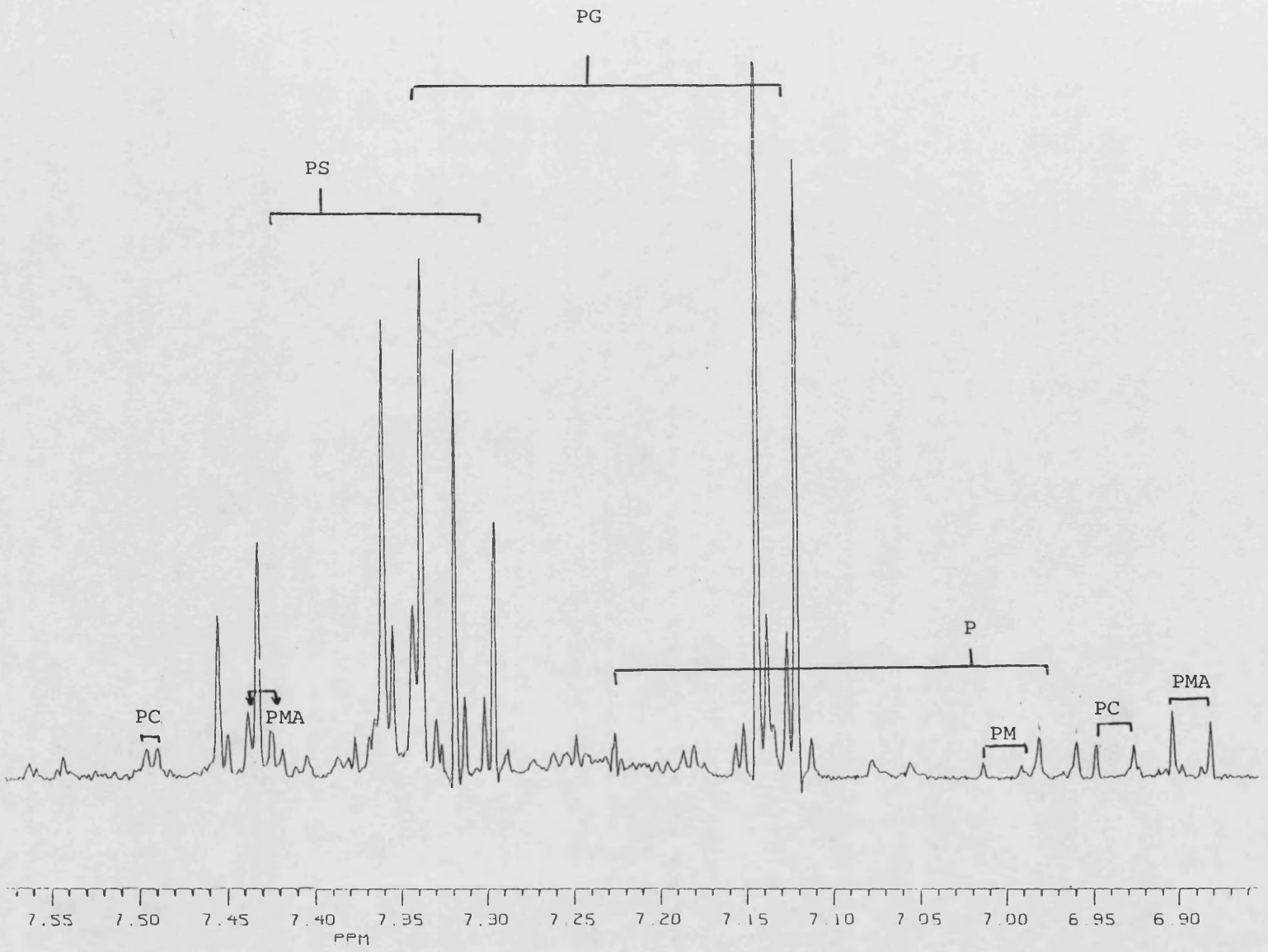
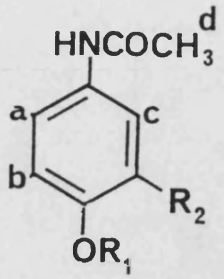
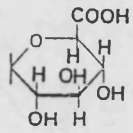


Fig. 3.2.2. AROMATIC REGION OF 400 MHz NMR SPECTRUM OF A URINE SAMPLE FROM A PATIENT WHO HAD TAKEN PARACETAMOL OVERDOSE AND HAD BEEN TREATED WITH PARVOLEX. CONDITIONS AS DESCRIBED IN Fig 3.1.1.

Table 3.2.1 Structures, 400 MHz ^{1}H NMR chemical shifts and coupling constants of paracetamol and its metabolites in human urine



Name, abbrev.	R Group	Chemical Shift, ppm	Coupling constant, J/Hz
Paracetamol, P	R_1 =-H R_2 =-H	H_a = 7.23 (d) H_b = 6.90 (d) H_d = 2.14 (s)	Jab = 8.7
Paracetamol glucuronide PG	R_1 =  R_2 =-H	H_a = 7.34 (d) H_b = 7.13 (d) H_d = 2.16 (s) H_{R1} = 5.11 (d) 3.60-3.94	Jab = 8.9
Paracetamol sulphate PS	R_1=-SO_3-K^+	H_a = 7.45 (d) H_b = 7.13 (d) H_d = 2.16 (s)	Jab = 8.9
Paracetamol mercapturic acid[a], PMA	R_1=-H R_2=-$SCH_2CH(COOH)NHCOCH_3$	H_a = 7.23 (d) H_b = 6.93 (d) H_c = 7.42 (d) H_d = 2.14 (s) H_{R2} = 1.84 (s) 3.28 (ABX), 4.30 (X)	Jab = 8.7 Jac = 2.1
Paracetamol L-cystein[a], PC	R_1=-H R_2=-$SCH_2CH(COOH)-NH_2$	H_a = 7.26 (dd) H_b = 6.99 (d) H_c = 7.57 (d) H_d = 2.15 (s) H_{R2} = 3.35 (ABX), 3,99 (X)	Jab = 8.7
Methoxy paracetamol[a], PM	R_1=-H R_2=-O-CH_3	H_a = 6.83 (dd) H_b = 6.90 (d) H_c = 7.08 (d) H_d = 2.15 (s) H_{R2} = 3.85 (s)	Jab = 8.4 Jac = 1.9

[a] From Bales' data[94]

s = singlet, d = doublet, dd = doublet of doublets, AB = second order AB multiplet

tables 3.2.2-3.2.8 (NMR results are not available in table 3.2.7 and 3.2.8) and are shown graphically in fig. 3.2.3 - 3.2.5. The concentrations of P and its metabolites are shown in table 3.2.9 - 3.2.11. Quantification of P,PG, PS and PAC by 400 MHz ^{1}H NMR was based on integrations of their acetyl methyl singlets (δ = 2.14, 2.16, 2.17 and 1.85 ppm respectively) compared to the methyl resonance of added valine (δ = 1.0 ppm) or DSS (δ = 0 ppm). The reliability of the quantification method was verified by linear correlation between the integrations within the concentration range and good recoveries (see section 2.10.1.4).

P and its metabolites were monitored by 400 MHz ^{1}H NMR. It can be seen from fig. 3.2.3 - 3.2.5 that P, PG and PS were high initially, followed by an increase in PG and PS after treatment with methionine or Parvolex (N-acetylcysteine). ^{1}H NMR spectra of urine from patients showed P, PG, PS, PMA and methoxy paracetamol (PM) resonances in earlier samples, followed by decreases of P and PM, increases of PG and PS and appearance of paracetmaol cysteine (PC). Table 3.2.12 lists the structures and chemical shifts of methionine and parvolex (N-acetylcysteine) in urine. Free methionine was not observed in the spectra, but N-acetyl cysteine resonances were detected, although no attempt was made to quantify them. Also, endogeneous metabolites were monitored to observe alterations from the normal pattern which might possibly occur. In most of ^{1}H NMR spectra of urine from patients taken P overdose, pyruvate and lactate resonances increased (see fig. 3.2.1 and 3.2.2), whilst hippurate, acetoacetate, β-hydroxybutyrate, glycine, L-alanine and citrate appeared to vary. In one patient, L-alanine completely disappeared and pyruvate increased enormously.

Table 3.2.2 Clinical events and mg of urinary paracetamol and its metabolites excreted from a patient (P1, male, 30 yr) after paracetamol overdose following treatment with Parvolex.

Time h	Event	Volume of urine, ml	mg excreted of P and its metabolites[a]									
			HPLC			NMR				SPEC		
			P	PG	PS	P	PG	PS	PMA	P	PG	PS
02.00	48 tablets of paracetamol ingested											
11.30	Admitted to Casualty Dept.											
13.00	Gatric wash-out											
	Plasma paracetamol (0.26 mg/ml)											
14.00	Admitted to C.P.U.											
14.40	5% Dextrose administered (700 ml)											
	Parvolex administered (8.7 g)											
14.50	Parvolex administered (2.9g)											
17.30		200	359.8	1785.4	413.8	298.2	1747.8	258.6	50.2	573.4	1721.4	258.6
19.00	5% Dextrose administered (1000 ml)											
	Parvolex administered (5.8g)											
02.30		475	288.3	3363.0	1271.1	315.4	3235.2	882.1	179.1	893.5	3339.7	882.1
08.45		500	59.5	1101.5	557.0	67.5	1079.5	490.5	128.5	318.0	1527.0	490.5
11.30		650	18.2	155.3	72.8	NM[b]	111.1	39.6	15.6	53.9	117.6	39.6
01.30		2150	32.2	326.8	219.3	NM	268.7	86.0	43.0	361.2	421.4	86.0
Total of individual metabolites (mg)			758.0	6732.0	2534.0	681.1	6442.3	1756.8	416.4	2200.0	7127.1	1756.8
	(%)[c]		7.6	67.1	25.3	7.3	69.3	18.9	4.5	19.8	64.3	15.9
Total recovery (mg)[c]			10024.0			929.6				11083.9		

[a] Expressed as mg of P equivalents
[b] NM = not measured
[c] Based on total P, PG and PS in HPLC and SPEC, and P, PG, PS and PMA in NMR

Table 3.2.3 Clinical events and mg of urinary paracetamol and its metabolites excreted from a patient (P2, female 27 yr) after paracetamol overdose following treatment with methionine

Time h	Event	Volume of urine, ml	mg excreted of P and its metabolites[a] HPLC P	HPLC PG	HPLC PS	NMR P	NMR PG	NMR PS	NMR PMA	SPEC P	SPEC PG	SPEC PS
23.00	49 tablets of paracetamol ingested											
00.10[b]	Admitted to Casualty Dept.											
00.30	Gatric wash-out											
	Methionine administered (2.5g)											
03.00	Plasma paracetamol (0.046 mg/ml)											
03.0		325	95.2	791.0	318.8	95.5	671.1	309.4	18.5	265.8	877.8	347.4
05.00	Methionine administered (2.5g)											
08.40		160	5.3	55.0	44.3	NM[c]	51.4	38.4	4.5	31.2	71.7	13.9
12.30		100	2.2	37.0	30.1	NM	34.5	30.2	6.1	30.4	36.5	48.0
Total of individual metabolites (mg)			102.7	883.0	393.2	95.5	757.0	378.0	29.1	327.4	986.0	409.3
	(%)[d]		7.5	64.0	28.5	7.6	60.1	30.0	2.3	19.0	57.2	23.8
Total recovery (mg)[d]			1378.9			1259.6				1722.7		

[a] Expressed as mg of paracetamol equivalents
[b] 25[h] h 10 min after the previous time
[c] NM = not measured
[d] Based on total P, PG and PS in HPLC or SPEC and P, PG, PS and PMA in NMR

Table 3.2.4 Clinical events and mg of urinary paracetamol and its metabolites excreted from a patient (P3, male 43 yr) after paracetamol overdose following treatment with methionine and Parvolex

Time h	Event	Volume of urine, ml	mg excreted of P and its metabolites[a]									
			HPLC			NMR				SPEC		
			P	PG	PS	P	PG	PS	PMA	P	PG	PS
unknown	Time of overdose paracetamol ingested											
17.30	Admitted to Casualty Dept.											
18.00	Gatric wash-out											
19.00	Admitted to C.P.U. Methionine administered											
21.00	Plasma paracetamol (0.254 mg/ml)											
21.25		900	386.1	632.7	204.3	331.2	562.5	180.9	18.9	447.3	774.9	ND[b]
22.00	5% Dextrose administered (700 ml) Parvolex administered (9.9g)											
22.15	Parvolex administered (3.3g)											
23.50		400	168.4	511.2	217.6	177.6	458.8	180.0	24.4	212.4	455.2	135.2
03.00	5% Dextrose administered (1000 ml) Parvolex administered (6.6g)											
09.30		900	436.5	4473.0	2718.0	381.6	3997.8	2072.7	206.1	1031.4	4278.6	2633.4
18.30		650	141.0	2493.4	1061.4	160.5	2295.1	1170.6	118.9	551.8	2607.8	1559.3
Total of individual metabolites (mg)			1132.0	8110.3	4200.9	1050.9	7314.2	3604.2	368.3	2242.5	8116.5	4327.9
		(%)[c]	8.4	60.3	31.2	8.5	59.3	29.2	3.0	15.3	55.3	29.5
Total recovery (mg)[c]			13443.2			12337.6				14686.9		

[a] Expressed as mg of paracetamol equivalents
[b] ND = not detected
[c] Based on total P, PG and PS in HPLC or SPEC, and P, PG, PS and PMA in NMR

Table 3.2.5 Clinical events and mg of urinary paracetamol and its metabolites excreted from a patient (P4, female 74 yr) after paracetamol overdose following treatment with methionine and Parvolex

Time h	Event	Volume of urine, ml	mg excreted of P and its metabolites[a]									
			HPLC			NMR				SPEC		
			P	PG	PS	P	PG	PS	PMA	P	PG	PS
unknown	Time of overdose paracetamol ingested											
11.20	Admitted to Casualty Dept.											
12.50	Gatric wash-out											
19.00	Admitted to C.P.U. Plasma paracetamol (0.4 mg/ml) Methionine administered (2.5 mg)											
14.00	5% Dextrose administered (800 ml)											
14.20	Parvolex administered (10.5g)											
14.35	Parvolex administered (3.5g)											
18.00	Parvolex administered (7.0g)											
22.0		375	168.7	2076.0	825.4	168.7	2077.1	844.5	52.5	187.5	2071.5	844.5
00.20		450	52.6	713.2	227.2	60.7	577.3	318.1	37.3	76.9	715.5	406.3
02.00		100	5.7	96.7	23.9	8.9	61.8	21.9	2.4	8.7	91.8	111.7
08.00		565	46.9	682.5	440.7	NM[b]	613.0	342.4	22.6	54.8	541.3	895.5
12.50		250	42.0	365.5	95.7	NM	506.5	101.2	14.0	60.2	329.2	511.0
15.30		125	3.1	54.5	16.5	NM	39.0	12.0	4.0	17.2	49.4	159.6
Total of individual metabolites (mg)			319.0	3988.4	1629.4	238.3	3874.1	1640.1	132.8	405.3	3798.7	2928.6
		(%)	5.4	67.2	27.4	65.8	27.9	2.3	5.7	0.8	52.1	41.1
Total recovery (mg)[c]			5936.8				5885.9			7132.6		

[a] Expressed as mg 0f paracetamol equivalents
[b] NM = not measured
[c] Based on total P, PG and PS in HPLC or SPEC, and P, PG, PS and PMA in NMR

Table 3.2.6 Clinical events and mg of urinary paracetamol and its metabolites excreted from a patient (P5, female) after paracetamol overdose following treatment with methionine

Time h	Event	Volume of urine, ml	mg excreted of P and its metabolites[a] HPLC P	HPLC PG	HPLC PS	NMR P	NMR PG	NMR PS	NMR PMA	SPEC P	SPEC PG	SPEC PS
unknown	20 tablets of Panadol ingested											
12.30	Admitted to Casualty Dept.											
13.00	Gatric wash-out											
14.00	Admitted to C.P.U.											
19.00	Methionine administered (2.5 mg)											
19.30		550	155.1	1959.1	529.6	154.0	1947.0	509.8	78.6	199.6	1898.6	968.0
22.20		610	106.7	1107.8	119.6	1061.4	193.4	193.4	23.8	132.4	982.7	511.2
22.45	Methionine administered (2.5g)											
02.45	Methionine administered (2.5g)											
02.50		600	39.0	924.0	199.2	30.6	886.2	231.0	48.0	66.0	741.6	588.6
Total of individual metabolites (mg)			300.8	3990.9	948.4	304.2	3894.6	934.2	150.4	398.0	3622.9	2067.8
		(%)[b]	5.7	76.2	18.1	5.8	73.7	17.7	2.8	6.5	59.5	34.0
Total recovery (mg)[b]			5240.1		5242.4	5283.4				6088.7		

[a] Expressed as mg paracetamol equivalents
[b] Based on total P, PG and PS in HPLC or SPEC; and P, PG, PS and PMA in NMR

Table 3.2.7 Clinical events and mg of urinary paracetamol and its metabolites excreted from a patient (P6, female, 35 yr) after paracetamol overdose following treatment with methionine

Time, h	Event	Volume of urine, ml	mg excreted of P and its metabolites[a] HPLC P	HPLC PG	HPLC PS	SPEC P	SPEC PG	SPEC PS
11.00	24 tablets of paracetamol ingested							
13.20	Admitted to Casualty Department							
14.00	Gastric wash-out							
15.00	Admitted to C.P.U.							
18.00	Methionine administered (2.5 g)							
19.30		200	655.2	1602.4	341.8	804.8	1411.0	975.4
22.00	Methionine administered (2.5g)							
01.00		200	128.6	1682.8	479.8	311.2	1889.6	911.6
02.00	Methionine administered (2.5g)							
08.45		260	47.1	1125.5	362.2	269.9	1066.8	1388.9
13.30		200	20.2	475.8	142.6	122.6	435.8	395.8
Total of individual metabolites (mg)			840.1	4886.5	1326.4	1508.5	4803.2	3671.7
	(%)[b]		11.9	69.3	18.8	15.1	48.1	36.8
Total recovery (mg)[b]			7053.0			9983.4		

[a] Expressed as mg of P equivalents
[b] Based on total P, PG and PS

Table 3.2.8 Clinical events and mg of urinary paracetamol and its metabolites excreted from a patient (P7, female) after paracetamol overdose following treatment with methionine

Time, h	Event	Volume of urine, ml	mg excreted of P and its metabolites[a] HPLC P	HPLC PG	HPLC PS	SPEC P	SPEC PG	SPEC PS
16.00	20-24 tablets of paracetamol ingested							
16.18	Admitted to Casualty Department							
16.40	Gastric wash-out							
17.20	Admitted to C.P.U.							
	Methionine administered (2.5g)							
18.05		340	266.2	746.0	181.2	399.8	682.0	356.0
20.00	Methionine administered (2.5g)							
21.10		100	73.8	233.4	539.8	97.6	225.5	596.9
24.00	Methionine administered (2.5g)							
04.00	Methionine administered (2.5g)							
07.00		100	16.5	311.2	100.1	62.0	365.9	199.4
Total of individual metabolites (mg)			356.5	1290.6	821.1	559.4	1273.4	1152.3
	(%)[b]		14.4	52.3	33.3	18.7	42.7	38.6
Total recovery (mg)[b]			2468.2			2985.1		

[a] Expressed as mg of P equivalents
[b] Based on total P, PG and PS

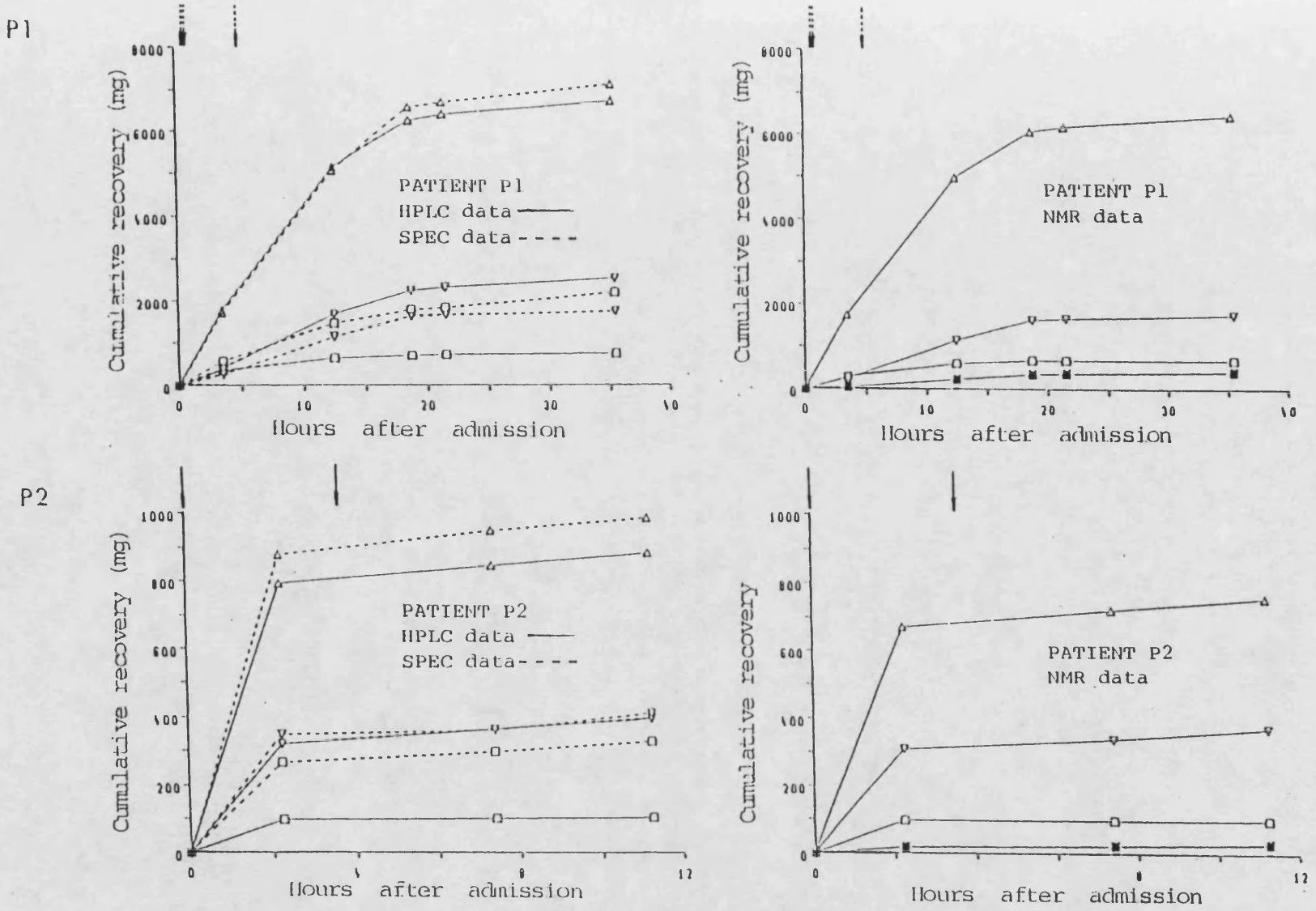


Fig. 3.2.1 Plots of cumulative recovery of paracetamol metabolites as a function of time in the urine of two patients whose details and events are given in Tables 3.2.1 and 3.2.2 measured by HPLC, SPEC , and NMR as described in the text. Arrows indicate administration of methionine ⟶ or N-acetylcysteine - - -➤. □ = paracetamol; ▽ = paracetamol sulphate; △ = paracetamol glucuronide; ■ = paracetamol N-acetylcysteine.

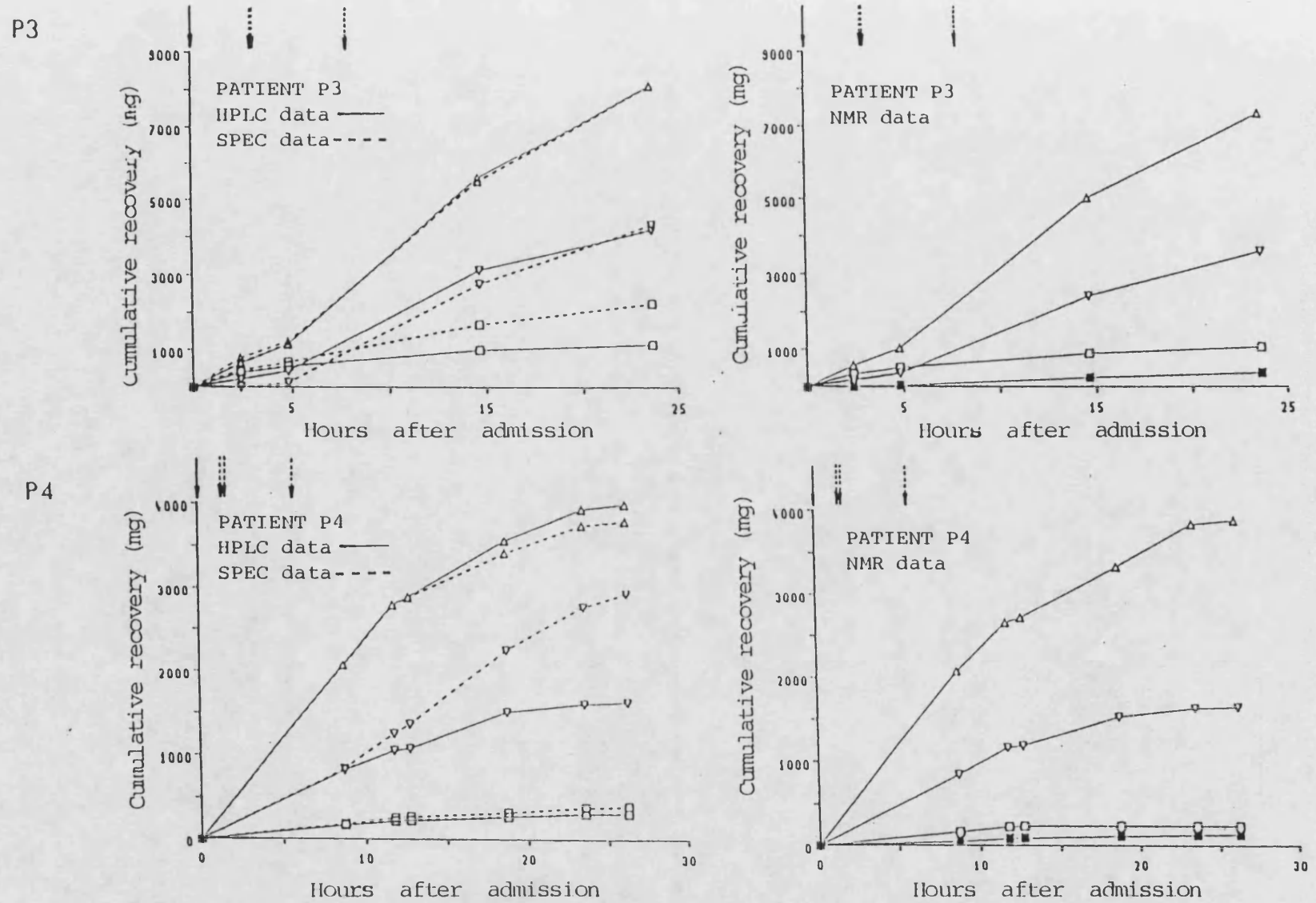


Fig. 3.2.2 Plots of cumulative recovery of paracetamol metabolites as a function of time in the urine of two patients whose details and events are given in Tables 3.2.3 and 3.2.4 measured by HPLC, SPEC, and NMR as described in the text. Arrows indicate administration of methionine ⟶ or N-acetylcysteine ⇢. □ = paracetamol; ▽=paracetamol sulphate; △=paracetamol glucuronide; ■ = paracetamol N-acetylcysteine.

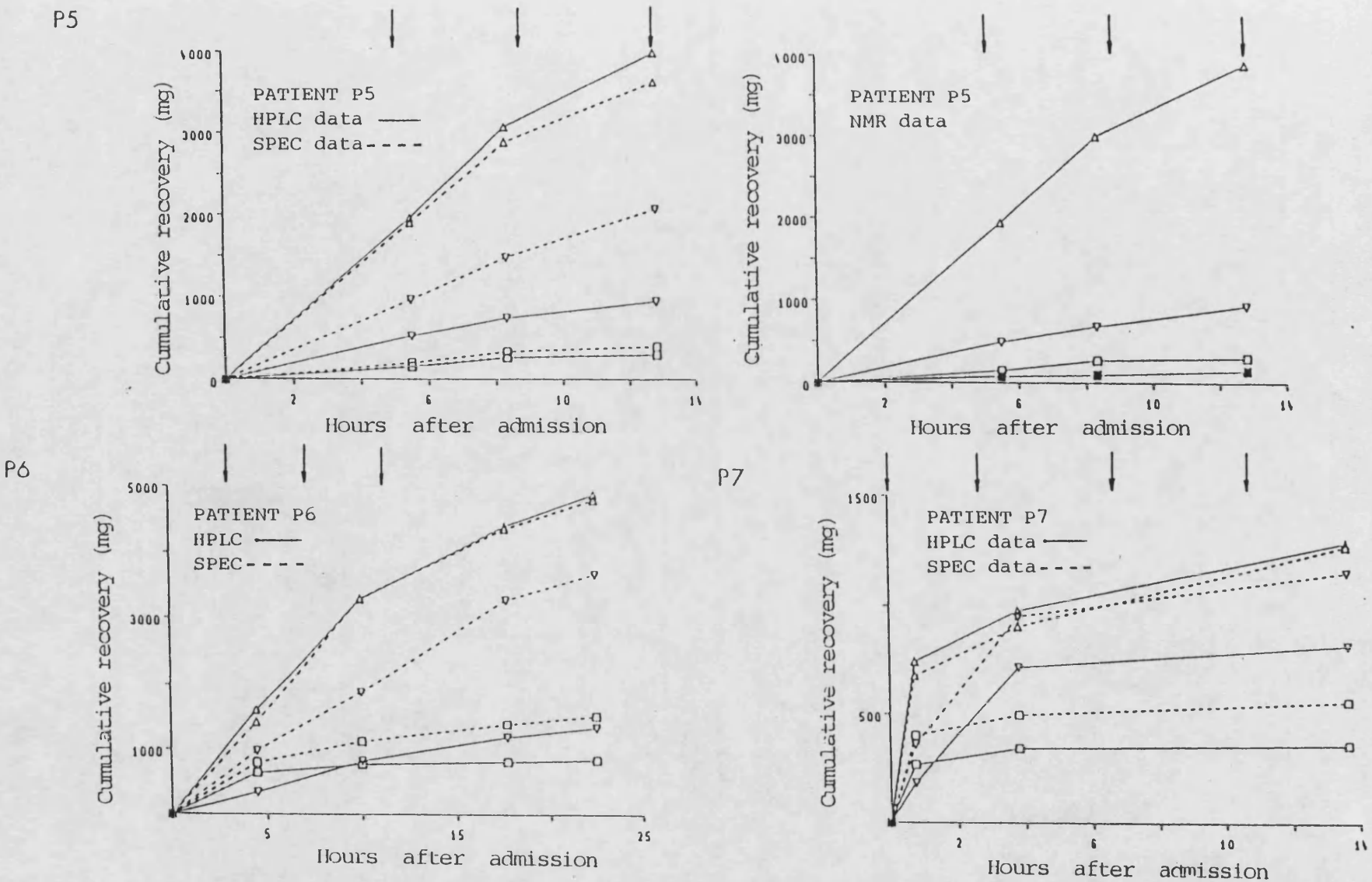


Fig. 3.2.3 Plots of cumulative recovery of paracetamol metabolites as a fuction of time in the urine of three patients whose details and events are given in Tables 3.2.5 - 7 measured by HPLC, SPEC, and NMR as described in the text. Arrows indicate the administration of methionine.
□ = paracetamol; ▽ = paracetamol sulphate; △ = paracetamol glucuronide; ■ = paracetamol N-acetylcysteine.

Table 3.2.9 Quantitation of paracetamol (P) from patients after paracetamol overdose, based on HPLC, NMR and SPEC methods

Subject	Time of Collection[a] h.min	Concentration (mM) P HPLC	NMR	SPEC	% Difference NMR/HPLC	SPEC/HPLC
P1	0 -3.30	11.90±0.21	9.86±0.30	18.96±0.53	82.9	159.3
male	3.30-12.30	4.01±0.09	4.39±0.38	12.44±0.14	109.5	310.2
30 yr	12.30-18.45	0.79±0.03	1.79±0.17	4.21±0.22	226.6	532.9
	18.45-21.30	0.18±0.01	NM	0.55±0.03	-	305.5
	21.30-35.30	0.10±0.01	NM	1.11±0.01*	-	1110.0
Pearson's[b]			r=0.999*	r=0.950		
Student's[c]			t=-0.24(NS)	t=2.53*		
P2	0 - 2.15	1.94±0.01	1.94±0.09	5.41±0.13	100.0	278.9
female	2.15- 7.25	0.22±0.01	NM	1.29±0.01	-	586.4
27 yr	7.25-11.15	0.14±0.01	NM	2.01±0.01	-	1435.7
Pearson's				r=0.980*(NS)		
Student's				t=3.03		
P3	0 -2.25	2.84±0.04	2.43±0.06	3.29±0.05	85.6	115.8
male	2.25-4.50	2.78±0.01	2.94±0.09	3.51±0.43	105.8	126.3
43 yr	4.50-14.40	3.21±0.02	2.80±0.10	7.58±0.26	87.2	236.1
	14.40-23.40	1.43±0.08	1.63±0.09*	5.62±0.13	114.0	393.0
Pearson's			r=0.917	r=0.036*(NS)		
Student's			t=-0.68(NS)	t=2.28		
P4	0 - 8.45	2.98±0.01	2.98±0.09	3.31±0.07	100.0	111.1
female	8.45-11.05	0.77±0.01	0.89±0.02	1.13±0.01	115.6	146.7
74 yr	11.05-12.45	0.38±0.01	0.59±0.01	0.57±0.04	155.3	150.0
	12.45-18.45	0.55±0.01	NM	0.64±0.03	-	116.4
	18.45-23.35	1.11±0.01	NM	1.59±0.08	-	143.2
	23.35-26.15	0.16±0.01	NM*	0.91±0.04*	-	568.7
Pearson's			r=0.999	r=0.975*		
Student's			t=0 (NS)	t=3.93		
P5	0 - 5.30	1.86±0.01	1.85±0.02	2.40±0.10	99.5	129.0
female	5.30- 8.20	1.16±0.01	1.30±0.01	1.43±0.10	112.1	123.3
	8.20-12.50	0.43±0.01	0.34±0.01	0.73±0.01	79.1	169.8
Pearson's			r=0.990(NS)	r=0.995*(NS)		
Student's			t=0.16(NS)	t=4.47		
P6	0 - 4.30	3.22±0.02		4.02±0.51		124.8
female	4.30-10.00	0.64±0.003		1.56±0.25		243.7
35 yr	10.00-17.45	0.18±0.001		1.04±0.12		577.8
	17.45-22.30	0.10±0.002		0.61±0.19*		610.0
Pearson's				r=0.994*		
Student's				t=8.64		
P7	0 - 0.45	0.78±0.01		1.18±0.01		151.3
female	0.45- 3.50	0.74±0.01		0.98±0.002		132.4
	3.50-13.40	0.17±0.01		0.62±0.01		364.7
Pearson's				r=0.956*(NS)		
Student's				t=5.61		

[a] Admission time to C.P.U. is taken as zero time; [b] HPLC versus NMR and SPEC
[c] Paired t-test, comparison with HPLC data; NM=not measured; * = $P<0.05$
NS = not significant

Table 3.2.10 Quantitation of paracetamol glucuronide (PG) from patients after paracetamol overdose, based on HPLC, NMR and SPEC

Subject	Time of Collection[a] h.min	Concentration (mM) PG HPLC	PG NMR	PG SPEC	% Difference NMR/HPLC	% Difference SPEC/HPLC
P1	0 -3.30	59.05±0.35	57.81±2.72	56.94±2.68	97.9	96.4
male	3.30-12.30	46.83±1.83	45.05±2.02	46.51±1.34	96.2	99.3
30 yr	12.30-18.45	14.57+0.33	14.28+0.36	20.20±2.68	90.8	138.6
	18.45-21.30	1.58±0.01	1.13±0.09	1.20±0.08	71.5	75.9
	21.30-35.30	1.00±0.04	0.83±0.08*	1.30±0.05*	83.0	130.0
Pearson's[b]			r=0.999	r=0.995		
Student's[c]			t=-0.12(NS)	t=0.18(NS)		
P2	0 - 2.15	16.10±0.14	13.66±0.43	17.87±0.51	84.8	111.0
female	2.15- 7.25	2.27±0.01	2.12±0.13	2.96±0.15	93.4	130.4
27 yr	7.25-11.15	2.45±0.34	2.28±0.03*	2.41±0.01*	93.1	98.4
Pearson's			r=0.999	r=0.998		
Student's			t=-1.20(NS)	t=1.50(NS)		
P3	0 -2.25	4.65±0.02	4.13±0.22	5.69±0.86	88.8	122.4
male	2.25-4.50	8.45±0.03	7.59±0.11	7.53±0.61	89.8	89.1
43 yr	4.50-14.40	32.88±0.16	29.38±0.11	31.45±1.06	89.4	95.7
	14.40-23.40	25.37±0.05	23.36±0.96*	26.54±3.30*	92.1	113.6
Pearson's			r=0.999	r=0.965		
Student's			t=-1.37(NS)	t=-0.05(NS)		
P4	0 - 8.45	36.62±0.09	36.53±1.50	29.26±2.50	99.8	79.9
female	8.45-11.05	10.48±0.09	10.52±0.62	9.86±0.83	100.4	94.1
74 yr	11.05-12.45	6.40±0.03	4.09±0.29	6.07±0.70	63.9	93.7
	12.45-18.45	7.99±0.03	7.18±0.50	6.34±0.93	89.9	79.4
	18.45-23.35	9.67±0.10	13.40±0.37	8.71±0.71	138.6	90.1
	23.35-26.15	2.88+0.02	2.06+0.10*	2.61±0.35*	71.5	90.6
Pearson's			r=0.985	r=0.988		
Student's			t=-0.41(NS)	t=-0.34(NS)		
P5	0 - 5.30	23.56±0.03	23.42±0.42	22.83±2.35	99.4	96.9
female	5.30- 8.20	12.01±0.03	10.65±0.30	11.32±0.99	88.7	94.3
	8.20-12.50	10.19±0.02	9.31±0.20*	8.18±0.67*	91.4	80.3
Pearson's			r=0.999	r=0.999		
Student's			t=-1.10(NS)	t=-1.23(NS)		
P6	0 - 4.30	8.01±0.04		7.06±0.66		88.1
female	4.30-10.00	8.41±0.02		9.48±0.76		111.4
35 yr	10.00-17.45	4.32±0.01		4.10±0.36		94.9
	17.45-22.30	2.38±0.001		2.18±0.62*		91.6
Pearson's				r=0.968		
Student's				t=-0.21 (NS)		
P7	0 - 0.45	2.19±0.01		2.01±0.05		91.8
female	0.45- 3.50	2.33±0.01		2.26±0.17		97.0
	3.50-13.40	3.11±0.02		3.66±0.28*		117.7
Pearson's				r=0.999		
Student's				t=0.41(NS)		

[a] Admission time to C.P.U. is taken as zero time; [b] HPLC versus NMR and SPEC
[c] Paired t-test, comparison with HPLC data; NM=not measured; * = $P<0.05$
NS = not significant

Table 3.2.11 Quantitation of paracetamol sulphate (PS) from patients after paracetamol overdose , based on HPLC, NMR and SPEC

Subject	Time of Collection[a] h.min	Concentration (mM) PS HPLC	PS NMR	PS SPEC	% Difference NMR/HPLC	% Difference SPEC/HPLC
P1	0 -3.30	13.69±0.47	8.55±0.41	15.07±1.00	62.4	110.1
male	3.30-12.30	17.70±0.28	12.28±0.81	21.92±2.82	69.4	123.8
30 yr	12.30-18.45	7.37±0.22	6.49±0.51	9.97±0.83	88.1	135.3
	18.45-21.30	0.74±0.02	0.40±0.02	1.05±0.14	54.0	141.9
	21.30-35.30	0.67±0.01	0.26±0.01	2.04±0.17*	38.8	304.5
Pearson's[b]			r=0.987*	r=0.994*		
Student's[c]			t=-1.27(NS)	t=3.15		
P2	0 - 2.15	6.49±0.03	6.30±0.23	7.07±0.28	97.1	108.9
female	2.15- 7.25	1.83±0.05	1.59±0.18	1.68±0.30	86.9	91.8
27 yr	7.25-11.15	1.99±0.06	2.00±0.08*	3.17±0.32	100.5	159.3
Pearson's			r=0.998*	r=0.971(NS)		
Student's			t=-1.49(NS)	t=3.11*		
P3	0 -2.25	1.50±0.07	1.33±0.08	ND	88.7	
male	2.25-4.50	3.60±0.06	2.98±0.08	4.84±0.33	82.8	134.4
43 yr	4.50-14.40	19.98±0.21	15.23±0.20	23.50±2.08	76.22	117.7
	14.40-23.40	10.80±0.37	11.91±0.17*	12.79±1.82*	110.3	118.4
Pearson's			r=0.965	r=0.999*		
Student's			t=-0.89(NS)	t=3.36*		
P4	0 - 8.45	14.56±0.08	14.90±0.97	14.90±1.00	100.0	102.3
female	8.45-11.05	3.34±0.03	4.68±0.27	5.97±0.90	103.0	140.1
74 yr	11.05-12.45	1.58±0.03	1.45±0.08	2.46±0.27	91.8	155.7
	12.45-18.45	5.16±0.02	4.01±0.19	5.24±1.00	70.7	101.6
	18.45-23.35	2.53±0.09	2.98±0.20	13.52±1.27	100.2	534.0
	23.35-26.15	0.87±0.01	0.63±0.03*	8.44±0.97	72.4	900.7
Pearson's			r=0.988*	r=0.590(NS)		
Student's			t=0.15(NS)	t=3.13*		
P5	0 - 5.30	6.37±0.05	6.13±0.44	11.64±1.07	96.2	182.7
female	5.30- 8.20	2.38±0.06	2.10±0.08	5.54±0.81	88.2	200.3
	8.20-12.50	2.20±0.06	2.55±0.12*	6.49±0.87	115.9	295.0
Pearson's			7r=0.987*	r=0.983(NS)		
Student's			t=-0.21(NS)	t=6.98*		
P6	0 - 4.30	1.71±0.03		4.88±0.66		285.0
female	4.30-10.00	2.40±0.03		4.56±0.76		190.0
35 yr	10.00-17.45	1.39±0.002		5.34±0.36		384.2
	17.45-22.30	0.71±0.01		1.98±0.62		278.9
Pearson's				r=0.660(NS)		
Student's				t=4.50*		
P7	0 - 0.45	0.53±0.05		1.05±0.05		198.1
female	0.45- 3.50	5.40±0.19		5.97±0.48		110.6
	3.50-13.40	1.00±0.05		1.99±0.01*		199.0
Pearson's				r=0.996*		
Student's				t=4.58*		

[a] Admission time to C.P.U. is taken as zero time; [b] HPLC versus NMR and SPEC
[c] Paired t-test, comparison with HPLC data; ND=not detected; * = $P<0.05$

NS = not significant

Table 3.2.12 Structures, 400 MHz ^{1}H NMR chemical shifts and coupling constants of methionine and Parvolex in human urine

Name, abbrev.	R Group	Chemical shift, ppm	Coupling constant, J/Hz
Methionine	$CH_3^aSCH_2^bCH_2^c\,\underset{NH_2}{\overset{COOH}{\overset{|}{CH^d}}}$	H_a = 2.12 (s) H_b = 2.63 (t) H_c = 2.16 (ABX) H_d = 3.83 (X)	J_{bc} = 7.5 Hz
Parvolex, or N-acetylcysteine	$^aH_3C\text{-}CO\text{-}NH\text{-}CH(COOH)\text{-}CH_2^bSH$	H_a = 2.04 (s) H_b = 3.24 (ABX) H_c = 4.38 (X)	

s = singlet, d = doublet, AB = second order AB multiplet

3.2.2 QUANTITATIVE ANALYSIS BY VISIBLE SPECTROPHOTOMETRY (SPEC)

Metabolite concentrations were calculated from calibration curves constructed for P (see section 2.8.2) based on the visible absorptions of P. Measurements of PG and PS were obtained after their enzymatic conversion to P by β-glucuronidase and sulphatase (completely converted to P as observed by HPLC) as previously described in section 2.8.2. Individual results and clinical details are given in tables 3.2.2-3.2.8 and are shown graphically in fig. 3.2.3-3.2.5. The concentrations of P and its metabolites are shown in tables 3.2.9-3.2.11. The reliability of the quantification method was verified by the linear correlation between the absorbance within the concentration range (see section 2.8.2).

3.2.3 QUANTITATIVE ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

P was identified on the basis of its retention time compared to standard P, i.e. 3.5 min. under the conditions described in section 2.9.2. Metabolite concentrations were calculated from the calibration curves constructed for P (see section 2.9.2) based on their peak height ratios to that of the internal standard (gentisic acid with retention time 5.1 min). Individual results and clinical details are given in tables 3.2.2-3.2.8 and are shown graphically in fig. 3.2.3-3.2.5. The concentrations of P and its metabolites are shown in table 3.2.9-3.2.11. The reliability of the quantification method was verified by linear correlation of the peak height ratio in the concentration range (see section 2.9.2).

3.2.4 COMPARISON OF RESULTS

Statistical analysis of SPEC, HPLC and NMR data (table 3.2.9-3.2.11) show that the concentration of P, PG and PS measured by ^{1}H NMR and that of PG measured by VIS are strongly correlated ($r > 0.98$) with those obtained on the same samples by HPLC. Since the acetyl methyl resonances among, P, PMA, PC and PM (δ = 2.14-2.15 ppm) were close, an attempt was made to get the best separation and integration of P resonance by resolution enhancement. Statistical analysis of the data (table 3.2.9) indicate that the concentrations of P measured by NMR were in good correlation (Pearson's) for 3 patients ($r>0.99$, $P<0.05$ for P1; $r>0.91$, $P<0.05$ for P3; and $r>0.99$, $P<0.002$ for P4) and not correlated for 1 patient (P4) with those obtained on the same samples by HPLC. Student's t-test for paired samples showed no

significant difference between P concentrations measured by the two methods.The concentrations of P measured by SPEC were found to be not correlated with those by HPLC in 4 patients (P2, P3, P5 and P7) out of 7 patients. The concentrations of PG (table 3.2.10) measured by 400 MHz ^{1}H NMR and SPEC were found to be in good correlation with those by HPLC in all patients ($r>0.99$, $P<0.05$ except for P4 where $r>0.98$, $P<0.001$ by NMR and for P6, $r>0.96$, $P<0.05$ by SPEC). The concentrations of PS (table 3.2.11) measured by NMR were in good correlation with those by HPLC ($r>0.99$, $P<0.002$ for P1; $r>0.99$, $P<0.05$ for P2; $r>0.96$, $P<0.05$ for P3; $r>0.98$, $P<0.001$ for P4 and $r>0.99$, $P<0.05$ for P5). Similarly, there was no significant difference of PS concentrations measured by NMR and HPLC. Measurement of PS concentrations by SPEC (table 3.2.11) indicate no correlation and significantly different values to those by HPLC in 5 patients out of 7 patients. The concentrations of PG and PS measured by SPEC were significantly higher and those of PS were significantly lower compared to their values measured by HPLC in most patients(table 3.2.9-3.2.11). The time courses of the urinary metabolic profile of P after overdose were followed and PG was the major metabolite, followed by PS (fig. 3.2.3-3.2.5).

3.3 ACEMETACIN ANALYSIS BY ^{1}H NMR

270 MHz ^{1}H spectra of lyophilized urine (freeze dried and redissolved in acetone-d_6: $^{2}H_2O$ (3:2 v/v) at 25 times the original concentrations) from a control subject and a volunteer taking acemetacin (see section 2.10.1.2) are shown in fig. 3.3.1A and 3.3.1B respectively. Homogated secondary irradiation of the residual water signal was applied and a total of 48 FIDs were collected. A Gaussian resolution enhancement function was applied

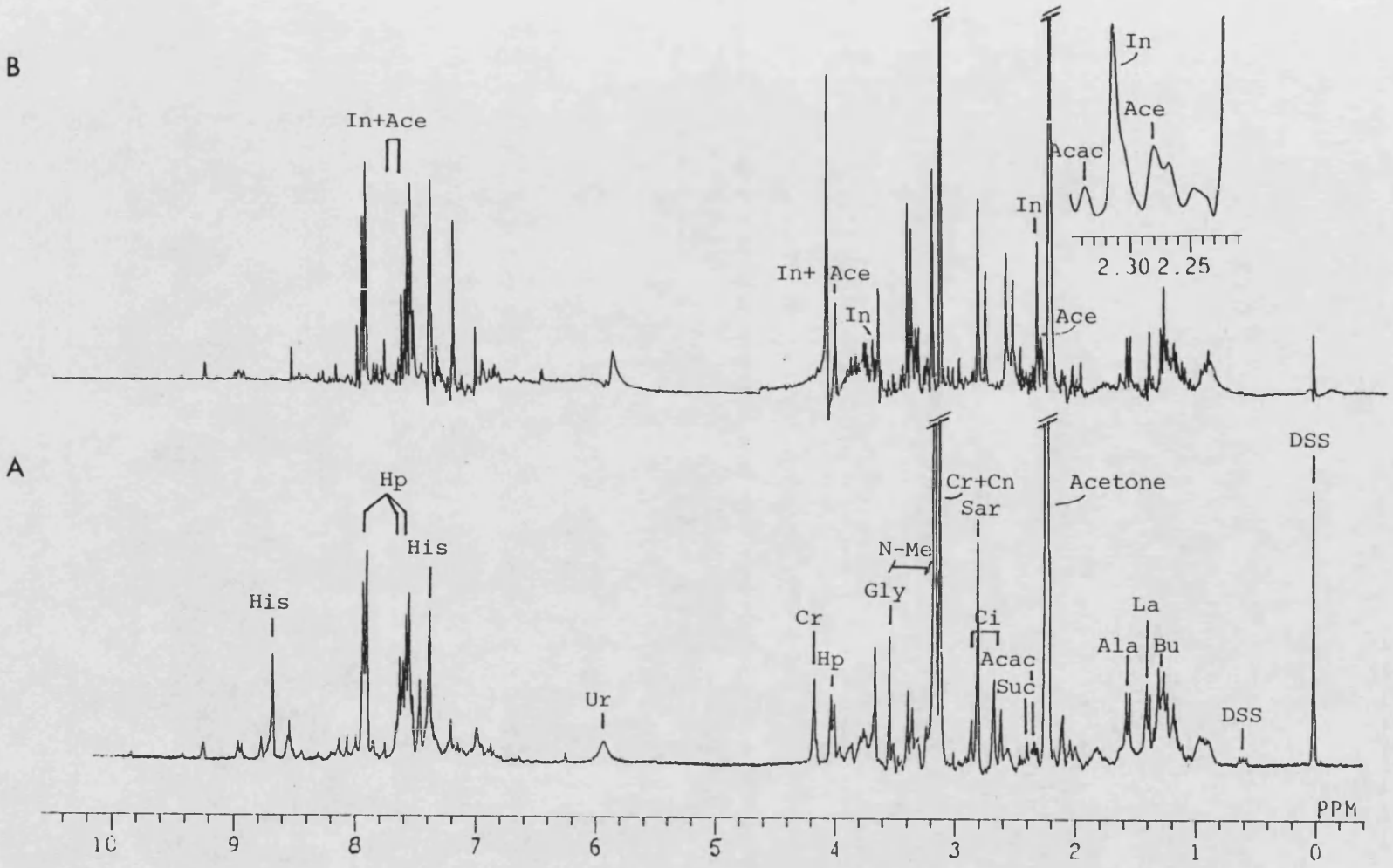
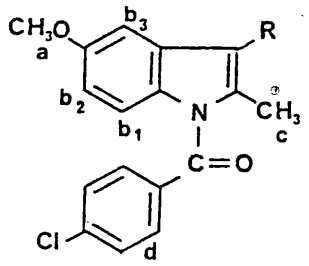


Fig. 3.3.1 270 MHz ^{1}H NMR spectra of lyophilized urine (to increase concentration about 25 x) in acetone-d_6 : D_2O (3:2 v/v) from (A) control (B) a volunteer after taking 180 mg/day acemetacin for 8 days. Resonances are labelled for 2,2-dimethyl-2-silapentane-5-sulphonate (DSS), β-hydroxybutyrate (Bu), lactate (La), L-alanine (Ala), acetone, citric acid, sarcosine (Sar), creatine (Cr), creatinine (Cn), N-Me group, glycine (Gly), hippuric acid (Hp), urea (Ur), histidine (His), indomethacin (IN) and acemetacin (ACE). For conditions see section 2.7.3 and 2.10.1.2.

Table 3.3.1 Structures, ^{1}H NMR chemical shifts and coupling constants of acemetacin (ACE) and indomethacin (IN) in Acetone-d_6 and $^{2}H_2O$ (3:2 v/v)



Name	R Group	Chemical shift ppm	Coupling Constant J/Hz
Acemetacin	$-CH_2^{e}COOCH_2^{f}COOH$	H_a = 3.87(s) H_{b1} = 7.00 (d) H_{b2} = 6.68 (dd) H_{b3} = 7.13 (d) H^{c} = 2.29 (s) H_d = 7.62 (d) 7.72 (d) H_e = 3.93 (s) H_f = 4.67 (s)	J_{b1b2} = 8.64 J_d = 7.56
Indomethacin	$-CH_2^{e}COOH$	H_a = 3.87 (s) H_{b1} = 6.98 (d) H_{b2} = 6.68 (dd) H_{b3} = 7.13 (d) H^{c} = 2.31 (s) H_d = 7.62 (d) 7.72 (d) H_e = 3.66 (s)	J_{b1b2} = 9.18 J_{b2b3} = 2.30 J_d = 8.64

(s) = singlet, (d) = doublet, (dd) = doublet of doublets

Table 3.3.2 ^{1}H NMR quantitation of acemetacin (ACE) and indomethacin (IN) in urine samples

Time[a]	Concentration (uM)					
	Subject 1		Subject 2		Subject 3	
	ACE	IN	ACE	IN	ACE	IN
Day 1, 2 h	27.11	109.27	24.23	128.63	20.81	105.19
Day 1, 4 h	25.68	104.77	NM	113.50	NM	125.13
Day 1, 6 h	NM[b]	99.39	NM	102.62	NM	93.79
Day 8, 2 h	16.73	97.19	6.25	27.61	NM	102.82
Day 8, 4 h	10.23	60.36	8.48	42.84	NM	45.39
Day 8, 6 h	6.68	59, 45	6.65	56.32	7.38	61.00

[a] 90 mg dose of acemetacin twice daily for 8 days.
[b] Not measured due to overlap with endogenous urinary components or low concentration.

prior to Fourier transformation. All the endogenous metabolites previously observed in unprocessed urine shown in fig. 3.1.1 were again detected with more intense signals and some chemical shift changes due to solvent and pH differences. Selective deuteration of the creatinine CH_2 group also occurred.

Table 3.3.1 shows the structures, ^{1}H NMR chemical shifts and coupling constants of acemetacin (ACE) and indomethacin (IN) spiked in blank urine samples and lyophilized as above. The methyl resonances of ACE and IN (at 2.29 ppm and 2.31 ppm respectively) were chosen and quantified using the weights of the paper traces of the spectra. The concentrations of urinary ACE and IN excreted from 3 human volunteers are shown in table 3.3.2. Endogenous metabolites in urine spectra were observed and found to vary between the volunteers. It was found that the urinary glycine resonance in the

NMR spectrum of one subject decreased on the 1st day and was constant during the 8th day of the study. Another subject showed no urinary glycine resonance in the NMR spectrum on the 8th day although glycine was present on day 1.

3.4 D-PENICILLAMINE ANALYSIS BY ^1H NMR

270 MHz single pulse ^1H NMR spectrum of "control" plasma obtained from EDTA-treated blood, are shown in fig. 3.4.1. The spectrum contains broad overlapping resonances with few sharp features. The most prominent of the broad resonances can be assigned to protons of mobile fatty acids (F_1-F_5)[102,111] (described in section 4.2) and to the broad envelope of protein resonances. In EDTA-plasma spectra where EDTA was used as an anti-coagulant, resonances from $CaEDTA^{2-}$ and $MgEDTA^{2-}$ were detected (fig. 3.4.2A and B). The other peaks assigned are a doublet of L-alanine (Ala,δ = 1.46 ppm), singlet of creatine (Cr, δ = 3.04 ppm), glycine (Gly,δ = 3.54 ppm) and doublet of α-anomeric proton of glucose (Glu,=δ 5.25 ppm).

Removal of plasma protein by ultra filtration (10000 molecular weight cut off) or by acid extraction with trichloroacetic acid (TCAA) gave well resolved spectra. For comparison to EDTA plasma spectrum, spectra of ultra filtered heparin plasma and serum are presented in fig. 3.4.3 and 3.4.4 respectively. Standard additions of some major component of plasma to EDTA plasma before acid extraction, freeze-drying and redissolving in ^2H_2O, and reference to the literature[112] were used to assign the peaks which are listed in table 3.4.1. The spin-echo spectrum of the same solution gave an aid to peak assignment due to its phase modulation.

Table 3.4.2 shows the structures and chemical shifts of D-penicillamine (PSH) and its metabolites (penicillamine disulphide - PSSP, penicillamine cysteine - PSSC, and S-methyl penicillamine - MPSH) in an acid extract of EDTA plasma following addition of standards to the plasma. 80 ml pooled EDTA plasma from 10 rheumatoid arthritis (RA) patients taking PSH treatment was extracted with acid to obtain PSH and its metabolites in the non-protein fraction of plasma. However, PSH and its metabolites were not detected in the 270 MHz ^{1}H NMR spectra, even after 40x concentration by freeze-drying and redissolution in $^{2}H_{2}O$ (fig. 3.4.2A and B). These spectra consist solely of several sharp peaks similar to those found in the control spectra from normal human EDTA plasma.

Table 3.4.1. STRUCTURE AND CHEMICAL SHIFTS OF SOME ENDOGENOUS COMPOUNDS IN PLASMA

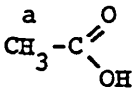
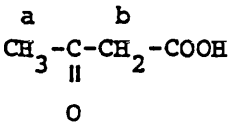
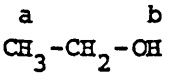
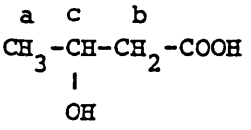
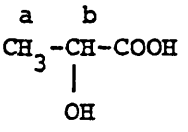
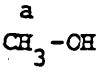
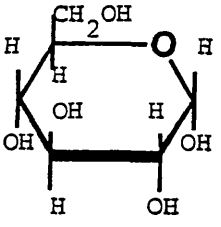
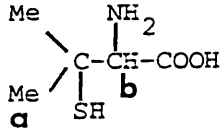
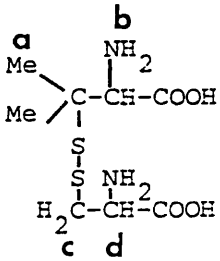
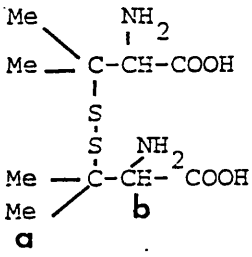
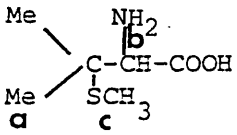
COMPOUND	ABBREV.	STRUCTURE	PPM
Acetate	Ac		a = 1.93(s)
Acetoacetate	Aca		a = 2.29(s) b = 3.45(s)
Acetone	Act		a = 2.24(s)
Ethanol	Et		a = 1.20(t) b = 3.67(q)
β-Hydroxybutyrate	Bu		a = 1.21(d) b = 2.29 - 2.44(m) c = 4.16(q)
Lactate	La		a = 1.34(d) b = 4.16(q)
Methanol	Me		a = 3.39(s)
Glucose	Glu		a = 3.2 - 4.0(m) b = 4.65(d) c = 5.25(d)
Lipid components (broad)	Li		0.9 - 1.3
Protein (broad)	Pro		aromatic residues 6 - 8

TABLE 3.4.2 STRUCTURE AND CHEMICAL SHIFTS OF D-PENICILLAMINE AND ITS METABOLITES

COMPOUND	STRUCTURE	CHEMICAL SHIFT (ppm)	COUPLING CONSTANT (Hz)
D-PENICILLAMINE (in phosphate buffer)		Ha = 1.51 Hb = 3.65 (s)	
PENICILLAMINE-CYSTEINE (in D_2O + NaOD)		Ha = 1.43 (s) 1.56 (s) Hc = 3.27 (d d) = 3.28 (d d) Hd = 4.04 (d d) Hb = 3.84 (s)	Jcd = 8;4 Jcc = 16
PENICILLAMINE-DISULPHIDE (in D_2O + NaOD)		Ha = 1.45 (s) = 1.55 (s) Hb = 3.72 (s)	
S-METHYL-PENICILLAMINE		Ha_1 = 1.307 (s) Ha_2 = 1.507 (s) Hb = 3.63 (s) Hc = 2.04 (s)	

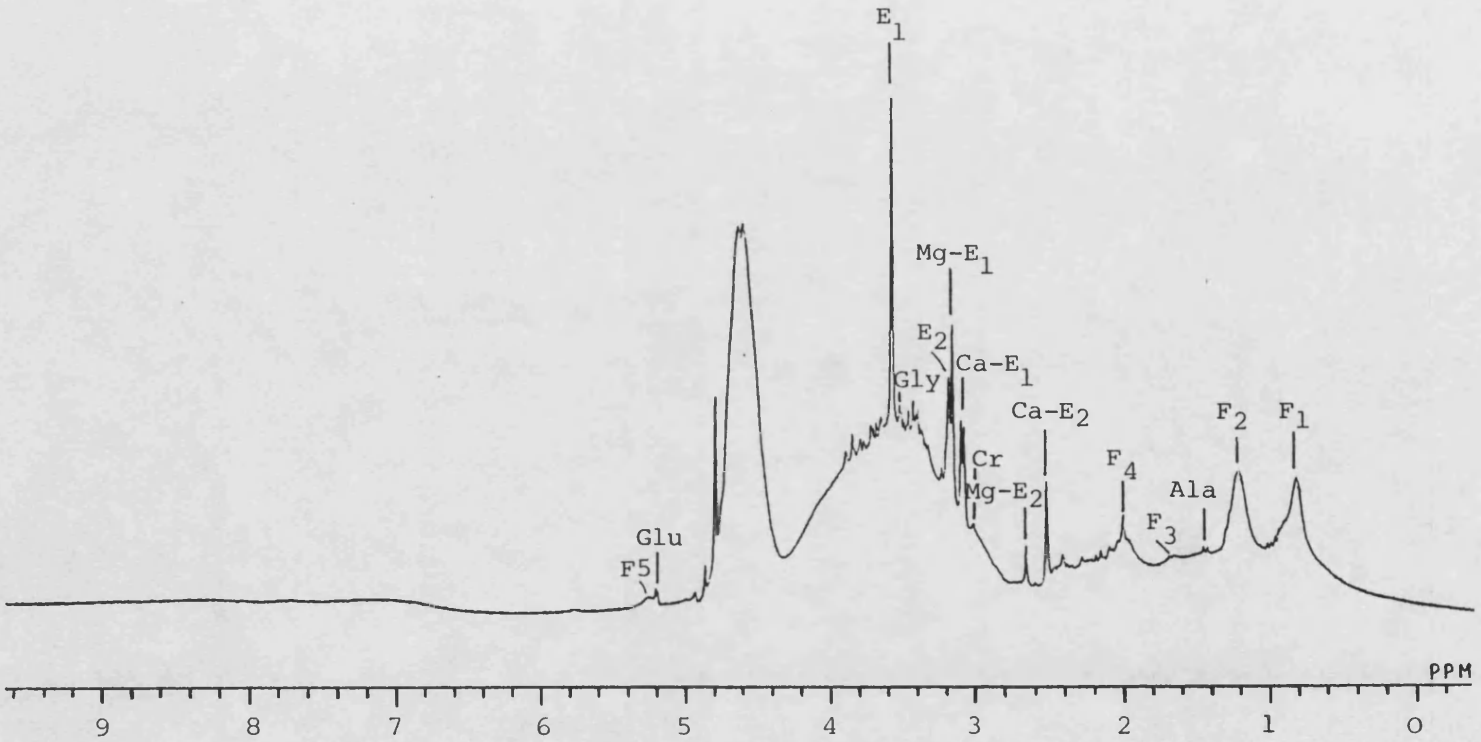


Fig. 3.4.1 270 MHz single pulse ^{1}H NMR spectrum of EDTA-plasma (EDTA-treated blood) containing 10% $^{2}H_2O$. Accumulation of 64 FID's at 24°C, using a 60° pulse, 5 s internal between pules (PD), and 16 K data points. The water resonances was suppressed by homogated secondary irradiation. An exponential function corresponding to a 1-Hz line broadening was applied. Abbreviations for peak assignments are given in table 3.4.1. DSS = 2,2-dimethyl-2-silapentane-5-sulphonate as reference. F_1-F_5 = mobile fatty acid resonances.

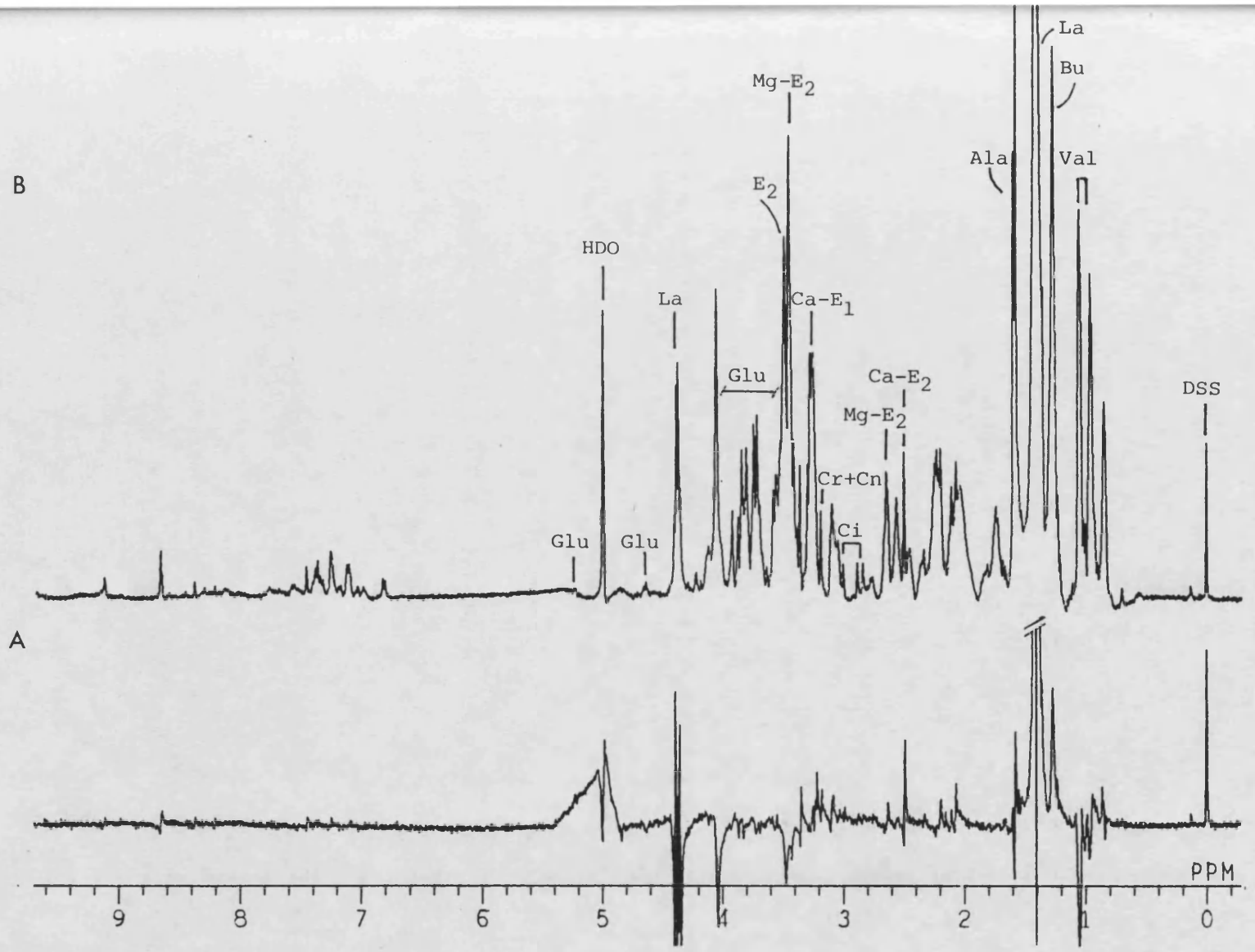


Fig. 3.4.2 270 MHz ^{1}H NMR spectra of acid extract of EDTA-plasma from patients taking D-penicillamine afterfreeze-drying and redissolved in $^{2}H_2O$ (to obtain 40 times of concentration strength) using (A) spin-echo sequence (τ = 60 ms) and (B) single pulses (normal acquisition). Other conditions are as described in fig. 3.4.1. Abbreviations for peak assignments are given in table 3.4.1. Some peaks have not yet been assigned.

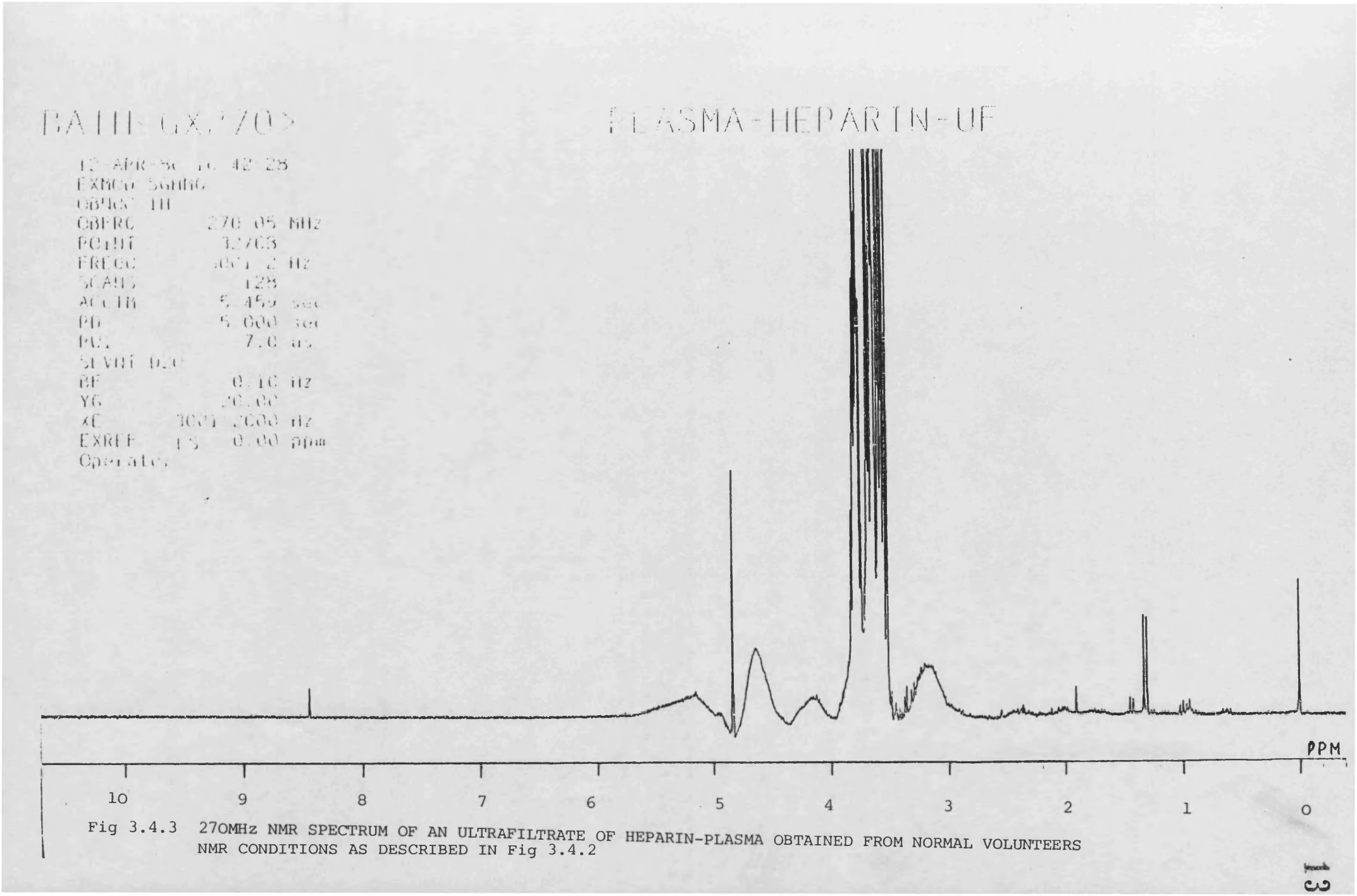


Fig 3.4.3 270MHz NMR SPECTRUM OF AN ULTRAFILTRATE OF HEPARIN-PLASMA OBTAINED FROM NORMAL VOLUNTEERS
NMR CONDITIONS AS DESCRIBED IN Fig 3.4.2

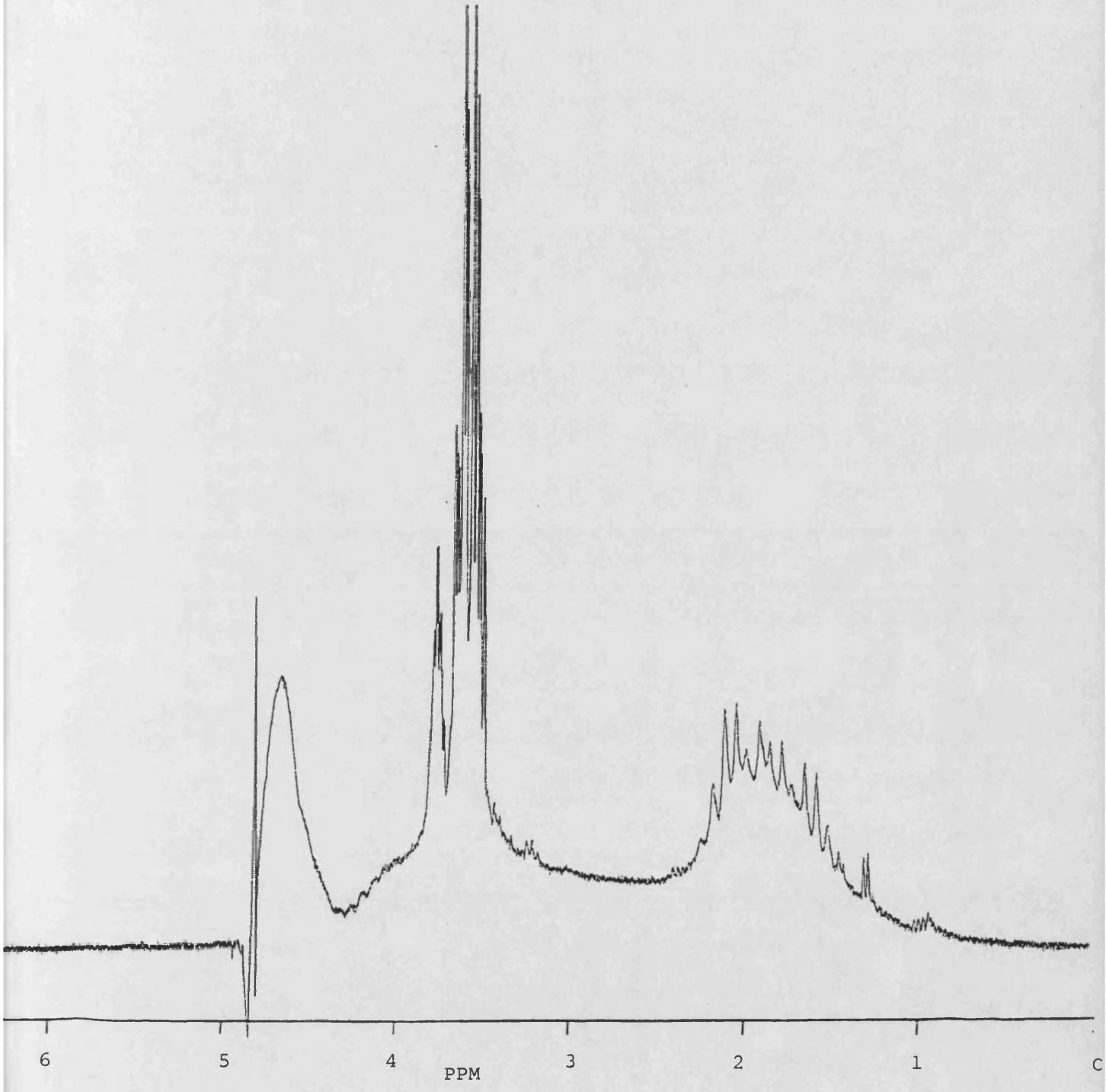


Fig. 3.4.4. 270MHz NMR SPECTRUM OF AN ULTRAFILTRATE OF SERUM OBTAINED FROM NORMAL VOLUNTEERS. NMR CONDITIONS ARE AS DESCRIBED IN Fig. 3.4.2

CHAPTER FOUR

RESULTS OF *IN VITRO* METABOLISM STUDIES BY PROTON NUCLEAR MAGNETIC RESONANCES SPECTROSCOPY

In vitro metabolism in rat liver preparations has been studied in the present work after enzyme induction with phenobarbitone or 3-methylcholanthrene. Enzyme induction by PB and 3MC was compared using spectral characteristics. The hepatic system was investigated by ^{1}H NMR using the 10,000 g liver fraction and enzyme induction was assessed by ^{1}H NMR using aminopyrine (DMAP) and daunorubicin (DA) as model substrates.

4.1 COMPARISON OF PHENOBARBITONE (PB) AND 3-METHYLCHOLANTHRENE (3MC) INDUCTION OF HEPATIC ENZYME SYSTEM

A summary of the effects of inducers on rat liver weight, liver protein concentration and microsomal cytochromes are given in table 4.1.1. The ratio of liver weight to body weight is greater in PB (0.040±0.002, mean+SD, n=4) and 3MC (0.036±0.002, n=4) treated rats than in control rats treated with saline (0.031±0.001, n=4) or corn-oil (0.029±0.002, n=4). A significant difference in protein content of 10,000 g liver fractions ($p<0.001$) was observed between PB-treated rats and control rats treated with saline (29.83 ± 1.34 and 25.49 ± 1.46 mg/g liver weight respectively) and between 3MC-treated rats and control rats treated with corn-oil (31.71 ± 1.67 and 24.92 ± 0.73 mg/g liver weight respectively).

A typical CO difference spectra of reduced cytochrome P-450/P-448 is given in fig. 4.1.1. The absorbance maximum, λ_{max} = 450nm, of the microsomal reduced cytochrome-CO complex from PB-treated rats was found to be at the same wavelength as for

control rats treated either with saline or corn-oil, with significantly ($P<0.001$) larger absorbances (corresponding to 2.324 ± 0.185, 0.844 ± 0.096 and 0.755 ± 0.073 nmoles/mg total protein respectively, see table 4.1.1). λ max in the CO difference spectrum of reduced cytochrome from the 3MC-treated rats changed from 450 nm to 448 nm in response to the induction with a significant increase in absorbance (corresponding to 1.525+0.204 nmoles/mg protein, $P<0.01$) compared to those of rats treated with saline or corn-oil.

A significant larger amount ($P<0.01$) of cytochrome b_5 in the liver from PB-treated rats was also found (0.986 ± 0.095 nmoles/g protein) compared to those of saline or corn-oil treated rats (0.663 ± 0.052 and 0.597 ± 0.052 nmoles/mg protein respectively). Cytochrome b_5 in the liver from 3MC treated rats (0.749 ± 0.067 nmoles/g protein) was not significantly greater than those of control rats. The two haemoproteins in liver microsomes, i.e. CO binding cytochrome P-450/P-448 and cytochrome b_5 (maximum at 450/448 and 428 nm respectively), could be distinguished from difference spectra (fig. 4.1.1).

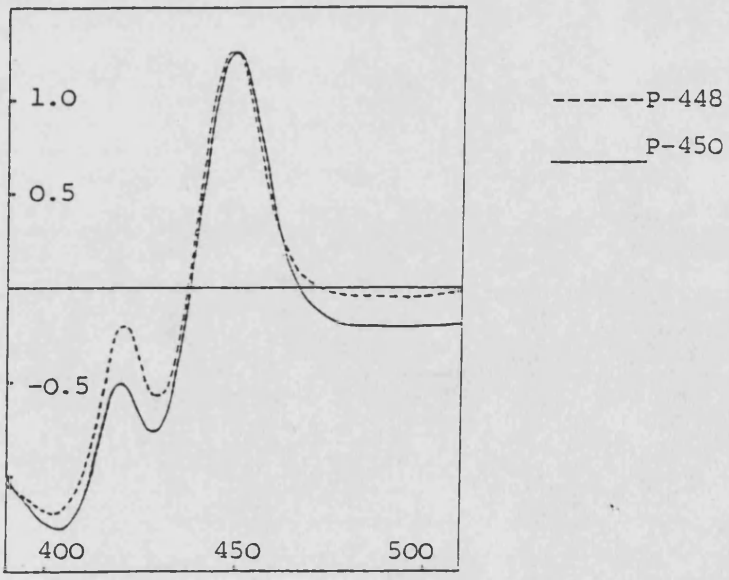


Fig. 4.1.1 CO difference spectra of reduced cytochromes after treatment with (A) phenobarbitone and (B) 3-methylcholanthrene

Table 4.1.1 The effects of PB and 3MC inducer on rat liver weight, 10,000 g liver fraction protein and microsomal cytochromes.

Treatment (n=4)	Body Weight (g)	Wet Liver Weight(g)	Liver Weight/ Body Weight ratio	Protein of 10,000xg Liver Fraction[a] (mg/g liver weight)	Microsomal CytochromP-450/P-448 (nmoles/mg protein)	Microsomal Cytochrome b_5 (nmoles/mg protein)
PB	333±26[b]	13.25±1.51	0.040±0.002	29.83±1.34	2.324±0.185	0.986±0.095
saline	331±21	10.23±0.52	0.031±0.001	25.49±1.46	0.844±0.096	0.663±0.052
3MC	334±24	11.99±1.22	0.036±0.002	31.71±1.67	1.525±0.204	0.749±0.067
Corn-oil	358±17	10.38±0.84	0.029±0.002	24.92±0.73	0.755±0.073	0.597±0.052

[a] Fraction of rat liver homogenate suspended in isotonic buffer (pH 7.4).
[b] Mean ± SD, $P<0.001$ compared to controls.

4.2 NMR ANALYSIS : CONTROL SPECTRA OF THE 10,000 G LIVER FRACTION

A single pulse and a spin-echo ^{1}H NMR spectra of the 10,000 g liver fraction (co-factors included, see section 2.10.2) are shown in fig. 4.2.1. The single pulse spectrum contains broad overlapping resonances with few sharp features. The most prominent of the broad resonances can be assigned to protons of unsaturated fatty acids[112,113,237,238] (F_1, terminal-CH_3; F_2, -$(CH_2)_n$-; F_3, -CH_2CH_2CO-; F_4, -CH_2CO-; F_5, -CH=CH). The spin-echo spectrum of the same suspension results in the elimination of many of the broad resonances. The sharper resonances due to protons with longer T_2 relaxation times still remain, i.e. the F_1 and F_2 protons of mobile fatty acids, probably part of triglycerides and phospholipids.

The other peaks in the NMR spectrum are the lower intensity resonances of some sharp singlet resonances (δ = 3.1-3.3 ppm) due to ^{+}N-$(CH_3)_3$ groups of the phospholipids, betaine or carnitine[103,237,238]. Glucose resonances at 3.40-3.95 ppm were identified as by spiking the 10,000 g liver fraction and were revealed as broadened peaks. The remainder of the signals arise from the co-factor, nicotinamide, in the aromatic region (δ = 7.5-9.0 ppm) but other co-factors, NADP and G6P, are not detected.

Fig. 4.2.2A and B show the spectra of an acid extract (with trichloroacetic acid, TCAA) of the 10,000 g liver fraction from PB-treated rats incubated without drugs added for 60 min. Even though the acid extraction caused the disappearance of signals from the most mobile fatty acids due to precipitation, some more resolved resonances (L-alanine at 1.56 pm, lactate at 1.41 ppm, β-hydroxybutyrate at 1.23 ppm and DSS at 0 ppm) were found due to the dissociation of these molecules from proteins.

Endogenous liver compounds were observed in the spectra of the

acid extract of the 10,000 g liver fraction (fig. 4.2.2A and B, table 4.2.1), but no attempt was made to quantify them accurately. However, estimation of the intensity of some endogeneous compounds with respect to the internal standard (DSS) can give an indication of concentration changes. Because of the broad signals and the suppression of resonance near the water signal, its was difficult to quantify the glucose signals (δ= 3.4 - 3.95 ppm). However, with careful phase correction it was possible to monitor the changes of peak intensities by estimating the peak in this region. Over a period of 120 min, the "control" 10,000 g liver fractions obtained from untreated rats and incubated without substrate showed a reduction in the intensity of the glucose signal and increases in those of lactate and L-alanine, while the β-hydroxybutyrate signal appeared to be constant. In the two controls, PB- and 3MC-treated, there was a 0.6-0.8 fold reduction of glucose and a 2.9-3.9 fold increase in L-alanine intensities after 120 min incubation. Lactate intensity increased over 120 min in all 10,000 g liver fractions, i.e. 4.5 fold in PB-treated and 1.6-2.4 fold in 3MC-treated and untreated rats.

4.3 NMR ANALYSIS: ASSESSMENT OF ENZYME INDUCTION USING AMINOPYRINE

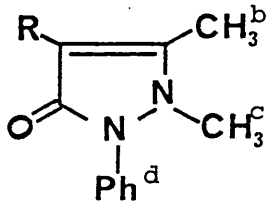
Aminopyrine (DMAP) is metabolised by cytochrome P-450. Its metabolism is shown in section 1.5.2.1. Induction of cytochrome P-450 will be expected to increase its rate of metabolism but that of cytochrome P-448 should have no effect. Aliquots of 10,000 g liver fraction, each containing 30 mg protein, were incubated in the presence of 2 mM DMAP (see section 2.10.2) and monitored by 270 MHz ^{1}H NMR at intervals over periods of up to 120 min. Fig. 4.3.1A and B show the spin-echo and single pulse NMR spectra

Table 4.2.1 Structures and 270 MHz ^{1}H NMR chemical shifts of some endogenous compounds of the acid extract of 10,000 g liver fraction

Compound	Abbrev.	Structure	^{1}H Chemical Shift/ppm[a]
Acetate	Ac	$H_3C-COOH$	2.09(s)
L-Alanine	Ala	$H_2N-\underset{CH_3}{CH}-COOH$	1.56(d)
Glucose	Glu	$H_2\underset{OH}{C}-\underset{3}{CH}-(CHOH)_4-O$	3.40-3.95
3-D-Hydroxybutyrate	Bu	$H_3C-\underset{OH}{CH}-CH_2-COOH$	1.23(d)
L-Lactate	La	$H_3C-\underset{OH}{CH}-COOH$	1.41(d)
$(CH_3)_3N^+$	NCH_3	$(H_3C)_3\overset{+}{N}-$	3.20-3.32
Pyruvate	Pyr	$H_3C-\underset{O}{C}-COOH$	2.66 (s)

[a] As measured from fig. 4.2.2 (s = singlet, d = doublet).

Table 4.3.1 Structures and 270 MHz ^{1}H NMR chemical shifts of aminopyrine (DMAP) and its metabolites in the acid extract of 10,000 g liver fraction, co factors and $MgCl_2$.



Name	Abbrev.	R group	Chemical shift, 1 ppm[a]
Dimethyl-4-aminoantipyrine or Aminopyrine	DMAP	$-N\overset{CH_3{}^a}{\underset{CH_3}{}}$	H_a = 3.340(s) H_a = 2.491(s) H_b = 3.350(s) H_c = 7.360-7.740(dd)
Monomethyl-4-aminoantipyrine	MMAP	$-N\overset{CH_3{}^a}{\underset{H}{}}$	H_a = 3.345(s) H_a = 2.454(s) H_b = 3.355(s)
4-Aminoantipyrine	AP	$-NH_2$	H_b = 2.418 (s) H_b = 3.230(s) H_c = 7.400-7.800(dd)

[a] As measured from fig. 4.3.1 (s = singlet, dd = doublet of doublets)

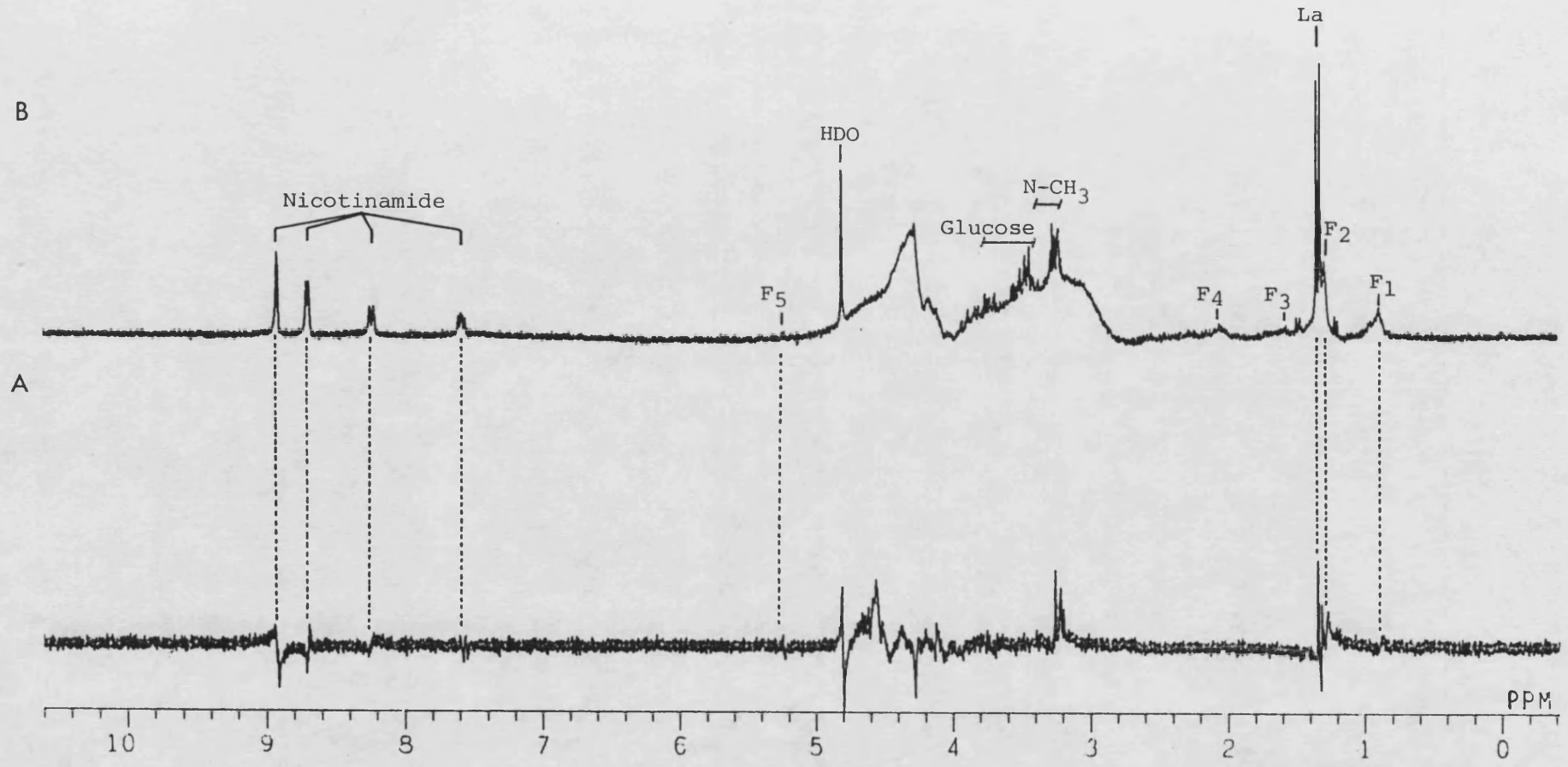


Fig. 4.2.1 270 MHz ^{1}H NMR spectra of Phenobarbitone treated 10,000 g liver fraction (contained 30 mg protein), cofactors and $MgCl_2$ (as described in section 2.10.2) in 0.1 M phosphate buffer (pH 7.4), obtained using (A) spin echo sequence (τ = 60 ms) and (B) single pulses (normal acquisition). Peaks have been assigned for nicotinamide, glucose, N-methyl groups (including choline head group of phospholipid at 3.2 ppm), lactate, mobile fatty acids (F_1, $-CH_3$; F_2, $-(CH_2)_n$; F_3, $-CH_2CH_2CO-$; F_4, $-CH_2CO-$; F_5, $-CH=CH-$); and, in B, a broad envelope of protein resonances. Note that F_1-F_5 resonances are suppressed due to phase modulation and relaxation effects.

Fig. 4.2.2 270 MHz ^{1}H NMR spectra of acid extract of Phenobarbital treated 10,000 g liver fraction (contained 30 mg protein) obtained using (A) spin echo sequence (τ = 60 ms) and (B) single pulses (normal acquisition). Resonances are labeled for 2,2-dimethyl-2-silapenthane-5-sulphonate (DSS), β-hydroxybutyrate (Bu), L-alanine (Ala), acetate (Ac), pyruvate (Pyr) and NADP in addition to those seen in fig. 4.2.1.

recorded after 30 min incubation of DMAP. Peak assignments of DMAP and its metabolites (table 4.3.1) were aided by spiking the 10,000 g liver fraction with standard metabolites. Fig. 4.3.2 shows the spectra from a series of experiments using 10,000 g liver fraction from PB-treated rats at different times (0,5,15,30, 60 and 120 min) after addition of DMAP. The intensity of the singlet resonance at 2.49 ppm due to the methyl protons of DMAP decreased with time. Appearance of the metabolites was first observed at 5 min (4-monomethylaminoantipyrine, MMAP), 15 min (4-aminoantipyrine,AP) and 60 min (unknown). However, no peak corresponding to the unknown metabolite appeared in the spectra of 3MC-treated or control rats within the 120 min incubation period. The resonances at the aliphatic region were at constant chemical shift, whereas those at the aromatic region were shifted possibly due to the slightly varied pH of samples.

Quantification of DMAP and its metabolites (MMAP and AP) was based on integrations of their methyl singlets (δ= 2.49, 2.45 and 2.42 ppm respectively) compared to those of the internal standard DSS (δ= 0 ppm) added. The results of these measurements over 30 min in different 10,000 g liver fractions (PB, saline, 3MC and corn-oil pretreatment) are shown in table 4.3.2. Statistical analysis of the data shows that the concentration of DMAP and metabolite MMAP in the 10,000 g liver fractions of PB- and 3MC-treated rats are strongly correlated ($r>0.96$, $P<0.02$, table 4.3.2) with those of control rats. Student's t-tests for paired samples were also performed. The concentration of DMAP in the liver of PB-treated rats was significantly lower ($P<0.01$) and those of metabolite MMAP was significantly higher ($P<0.02$) than those in the liver of saline, 3MC or corn-oil treated rats, whereas

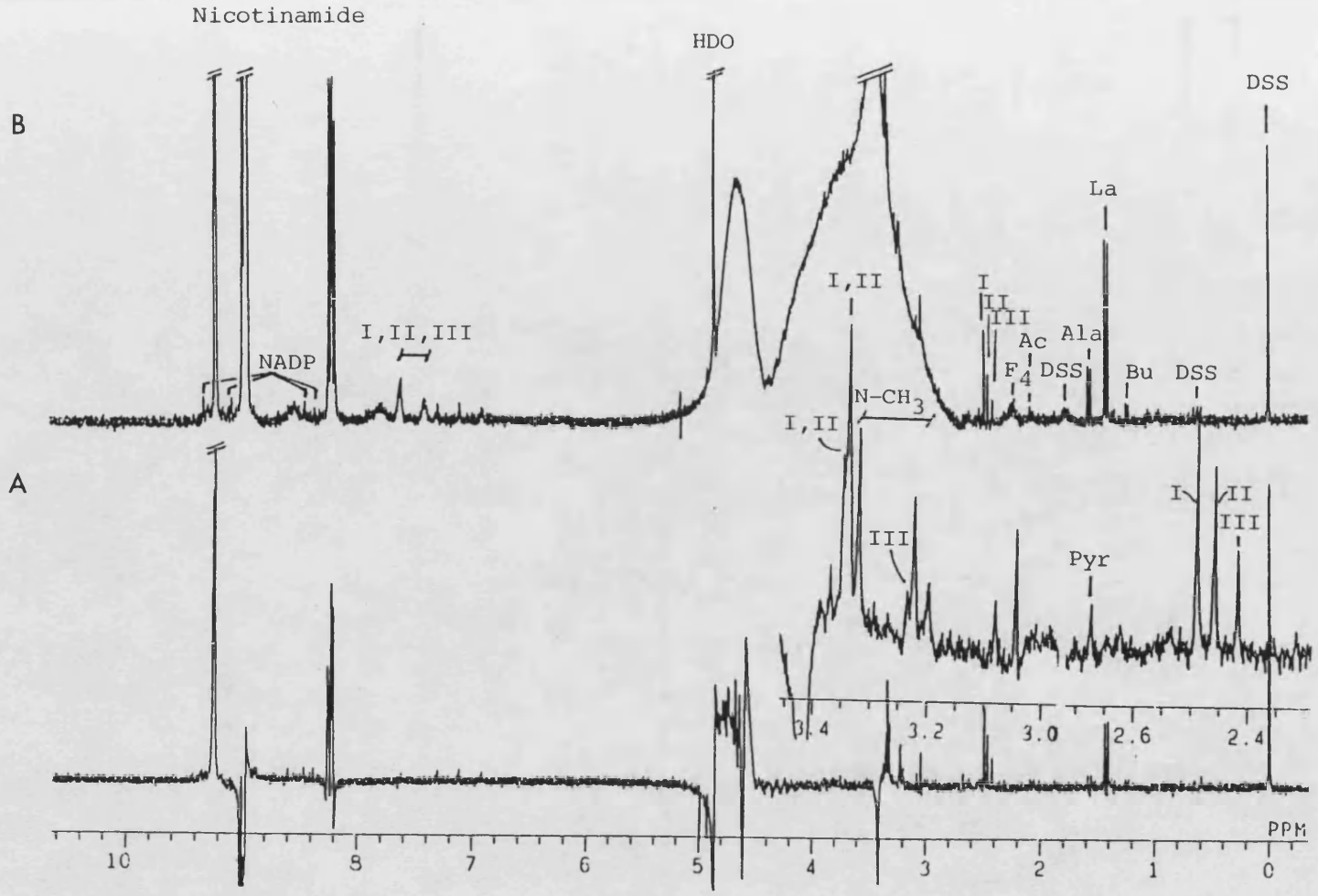


Fig. 4.3.1 270 MHz ^{1}H NMR spectra of acid extract of Phenobarbitone treated 10,000 g liver fraction (contained 30 mg protein) after 30 min incubation with 2 mM aminopyrine (DMAP) (A) a spin echo spectrum (τ = 60 ms); (B) single pulse NMR spectrum. Resonances are labelled for aminopyrine (I), monomethyl-4-aminoantipyrine (II) and 4-aminoantipyrine (III) in addition to those seen in fig. 4.2.2. Note that I, II and III resonances are suppressed due to phase modulation and relaxation effects. Inset: expansions of methyl region, 2.4-2.5 ppm and 3.2-3.4 ppm.

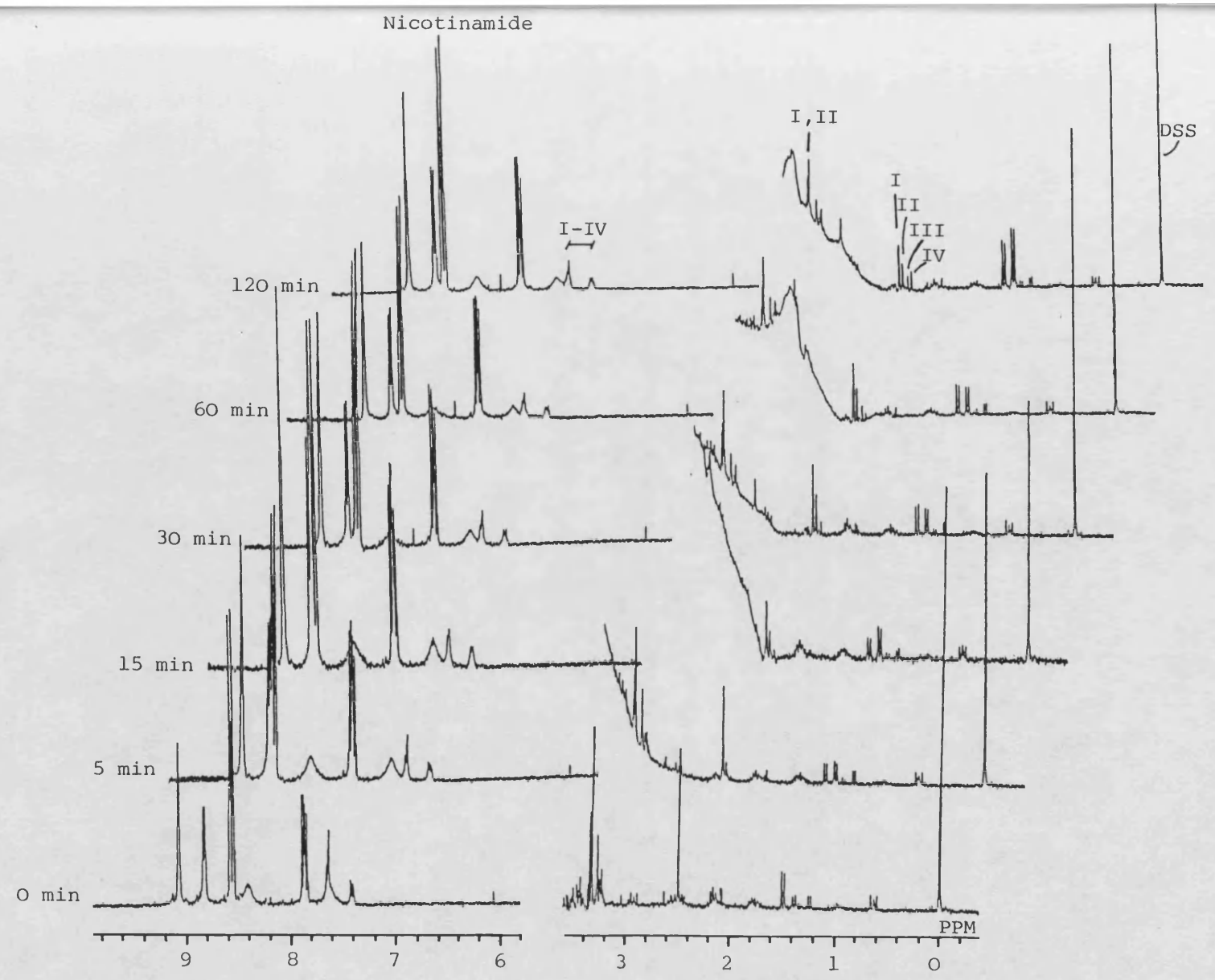


Fig. 4.3.2 A stacked plot showing the 270 MHz ^{1}H NMR spectra of acid extract of PB treated 10,000 g liver fraction after incubation with 2 mM aminopyrine (I) at 0.5,15,30,60 and 120 min. The reduction of I and the appearance of its metabolites (II, monomethyl-4-aminoantipyrine; III, 4-aminoantipyrine; and IV, unknown) can be seen. The slightly various pH's of the samples are reflected in the shifts of the resonances at aromatic region, whereas those aliphatic region do not change. Resonance is labeled for IV in addition to those seen in fig. 4.3.1.

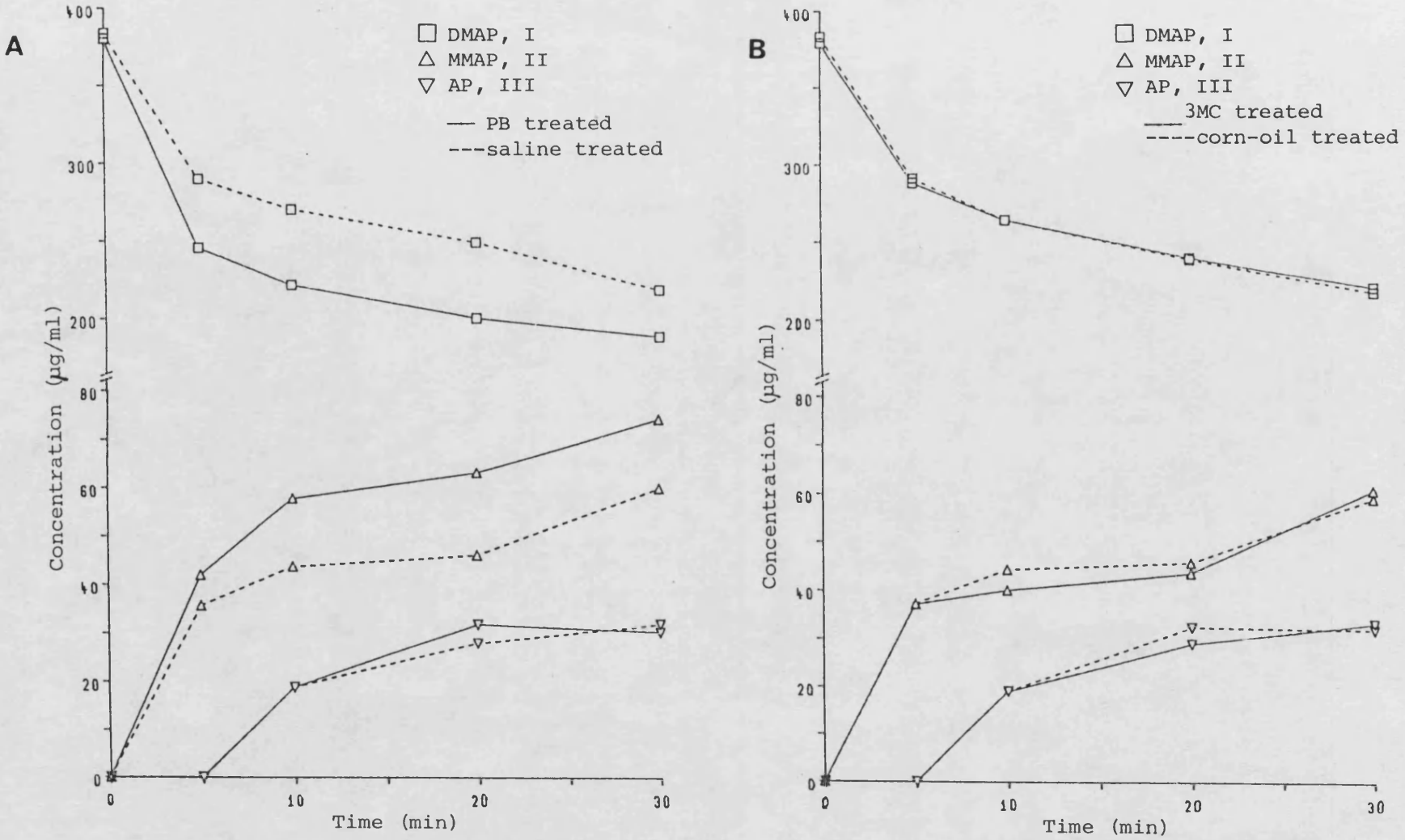


Fig. 4.3.3 Mean values of concentration vs time of aminopyrine and its metabolites, monomethyl-4-aminoantipyrine and 4-aminoantipyrine in (A) Phenobarbitone, saline, and (B) 3-methylcholanthrene and corn-oil treated 10,000 g liver fraction (each equivalent to 30 mg protein) as measured by 270 MHz ; 1_H

Table 4.3.2 Concentration of aminopyrine and its metabolites in control and PB or 3MC treated 10,000 g liver fraction (equivalent to 30 mg protein) as measured by ^1H NMR from integrations of methyl resonances (δ= 2.41-2.49 ppm) compared to that of DSS (δ= Oppm) in single-pulse experiments.

Inducer or Control	Incubation Time (minutes)	Concentration (µg/ml DMAA equivalent)[a] DMAP	MMAP	AP	Total (µg/ml)	Substrate Found (1%)[b]
PB	0	380.77 ± 3.94[c]	ND	ND	380.77	97.28
	5	245.96 ± 1.01	42.13 ± 0.32	ND	288.09	73.61
	10	221.96 ± 5.74	58.06 ± 1.92	18.98 ± 0.25	299.00	76.39
	20	201.00 ± 1.72	63.43 ± 1.21	31.97 ± 0.75	296.40	75.73
	30	189.19 ± 3.92	74.53 ± 1.51	30.39 ± 0.62	294.11	75.14
Pearson's[d]		r= 0.985 (P<0.01)	r=0.964 (P<0.02)	r= 0.911 (NS)		
Student's[e]		t=-9.81 (P<0.01)	t=5.70 (P<0.02)	t= 1.25 (NS)		
Saline	0	384.08 ± 0.38	ND	ND	384.08	98.13
	5	290.07 ± 4.06	35.71 ± 0.38	ND	325.78	83.23
	10	270.50 ± 1.89	43.88 ± 1.13	18.94 ± 0.31	333.32	85.16
	20	250.06 ± 3.68	46.47 ± 1.36	27.96 ± 1.03	324.49	82.90
	30	219.46 ± 2.87	60.23 ± 1.51	32.12 ± 0.83	311.81	79.67
3MC	0	379.56 ± 4.76	ND	ND	379.56	96.97
	5	288.87 ± 2.05	37.13 ± 0.35	ND	326.00	83.29
	10	264.94 ± 6.81	40.14 ± 1.08	19.14 ± 1.15	324.22	82.84
	20	241.24 ± 2.26	43.78 ± 0.66	29.04 ± 0.73	314.06	80.24
	30	222.52 ± 3.48	61.03 ± 1.43	33.15 ± 0.23	316.70	80.91
Pearson's		r=0.999 (P<0.001)	r= 0.977 (P<0.02)	r= 0.946 (NS)		
Student's		t=0.54 (NS)	t=-0.92 (NS)	t=-0.50 (NS)		
Corn-oil	0	383.50 ± 4.31	ND	ND	383.50	97.98
	5	291.27 ± 3.06	37.26 ± 1.69	ND	328.53	83.94
	10	264.81 ± 6.32	44.42 ± 0.43	19.18 ± 0.19	328.41	83.91
	20	239.63 ± 3.654	45.95 ± 0.91	32.45 ± 1.48	318.03	81.25
	30	219.23 ± 3.35	59.24 ± 0.88	31.83 ± 0.77	310.30	79.28

[a] DMAP, MMAP, and AP stand for aminopyrine, monomethyl-4-aminoantipyrine and 4-aminoantipyrine respectively.

[b] Initial concentration of aminopyrine is 391.40 µg/ml.

[c] Mean ± SD

[d] PB versus saline or 3MC versus corn-oil
ND = not detected

[e] Paired t-test between PB and saline, or 3MC and corn-oil
NS = not significant

the concentration of metabolite AP was the same in all liver preparations. The mean concentrations of DMAP, MMAP and AP in the 10,000 g liver fraction of various treated rats are plotted *vs.* time over a 30 min period in fig. 4.3.3.

A major difference between the ^{1}H NMR spectra of the 10,000 g liver fraction in the presence of 2 mM of DMAP and the controls was that the resonances for glucose increased in the former spectra, whereas for controls, resonances in this region decreased in intensity (see section 4.2). However, the increases in methyl doublets of L-alanine (δ=1.56 ppm) and lactate (δ=1.41 ppm) were found to be similar to those in control 10,000 g liver fractions from PB, saline, 3MC or corn-oil treated rats.

4.4 NMR ANALYSIS : ASSESSMENT OF ENZYME INDUCTION USING DAUNORUBICIN

Daunorubicin is metabolised in the liver by cytochrome P-450 and P-448 and its metabolic pathways were examined with different types of enzyme induction. Its metabolism is shown in section 1.5.2.2. Various 10,000 g liver fractions (i.e. from PB, saline, 3MC or corn-oil treated rats), each containing 30 mg protein, were incubated in the presence of 2 mM daunorubicin (DA) and monitored by 270 MHz ^{1}H NMR at intervals over a period of 30 minutes. Fig. 4.4.1, 4.4.2 and 4.4.3 show the spin-echo and single pulse NMR spectra of 10,000 g liver fraction from PB, 3MC and corn-oil treated rats recorded after 30 min incubation with DA. The spectrum after saline treatment is not presented but showed identical resonances to those after corn-oil treatment. Table 4.4.1 shows the peak assignments of DA which were aided by spiking the 10,000 g liver fraction with standard compound. Fig. 4.4.4A and B, and fig.

4.4.5A and B, show a series of the above experiments at different reaction times (0, 10 , 20 and 30 min) after the addition of DA using 10,000 g liver fraction from saline, PB, corn-oil and 3MC treated rats respectively. Of these four types of supernatants, that employing PB treatment gave the most rapid rate of DA metabolism as assessed by the decrease in the methyl signal intensity at 1.31 ppm. The appearance of metabolites could be seen in the spectrum of the 10 min incubation, i.e. DA_1 (δ=1.25 and 1.28 ppm) and DA_2 (δ =1.20 and 1.23 ppm). In the 10,000 g liver fraction from 3MC-treated rats, the spectrum showed one other metabolite, i.e. DA_3 (δ =1.06 and 1.09 ppm). No attempt was made to extract these metabolites and determine their structures due to the lack of time.

The DA preparation used in the present studies contained mannitol as a stabilizer, which gave signals in the range 3.86-3.93 ppm in the experimental conditions employed. Hence the glucose intensities at 3.4-3.95 ppm could not be monitored. However, the intensities of L-alanine signals over a 30 min incubation period with DA added were found to increase 2.8-3.5 fold. The lactate intensities over 30 min incubations were also found to increase , i.e. 3.4 fold in PB-treated and 2.1-2.3 fold in 3MC, saline and corn-oil treated rats.

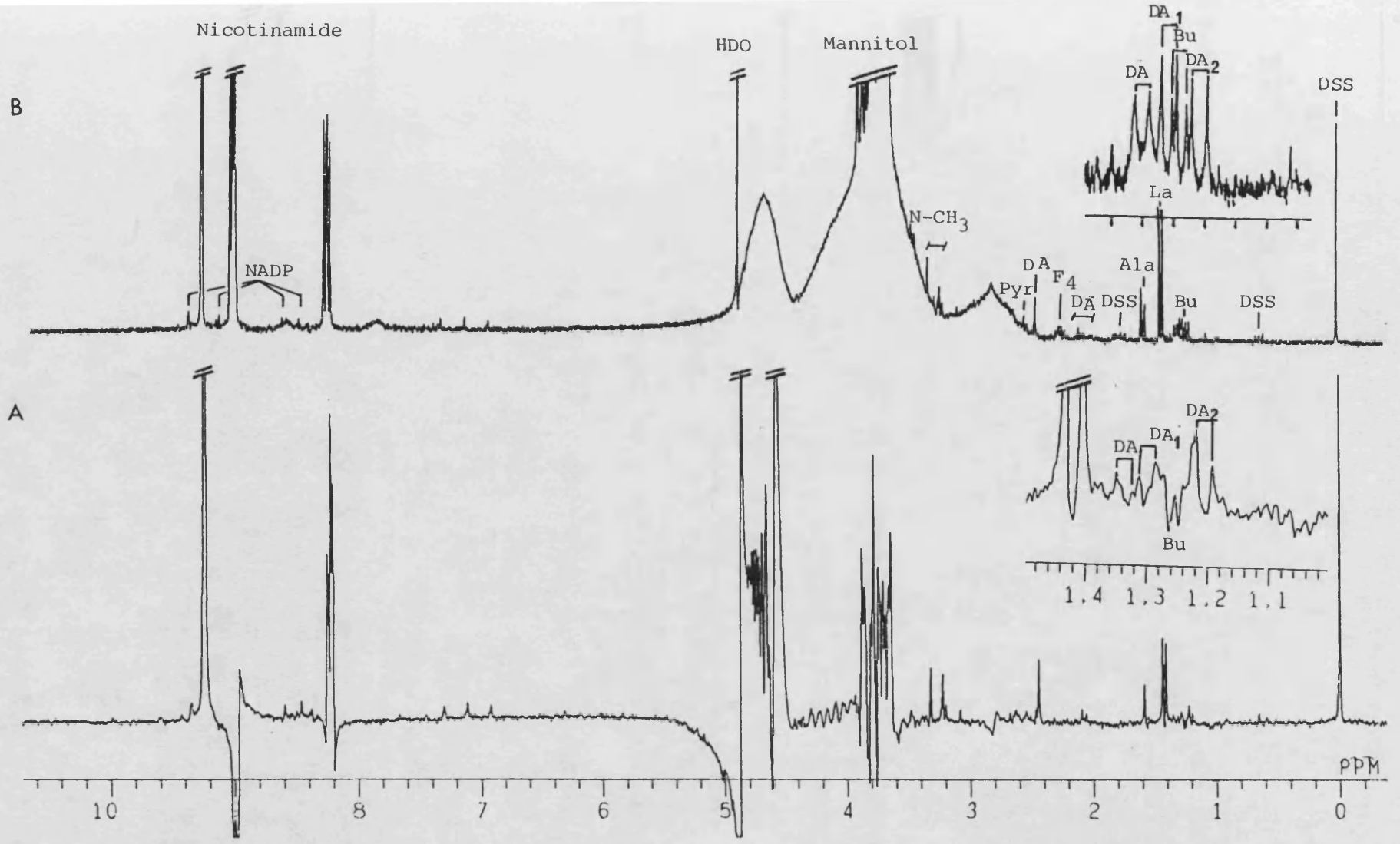


Fig. 4.4.1 270 MHz ^{1}H NMR spectra of acid extract of Phenobarbital treated 10,000 g liver fraction (contained 30 mg protein) after 30 min incubation with 2 mM daunorubicin (DA) (A) a spin echo spectrum (τ = 60 ms); (B) single pulse NMR spectrum. Resonances are labelled for DA and its metabolites (DA_1 and DA_2) in addition to those seen in fig. 4.2.2. Note that DA, DA_1 and DA_2 resonances (about 1.2-1.3 ppm) are suppressed due to phase modulation and relaxation effects. Inset: expansions of methyl region, 1.0-1.5 ppm.

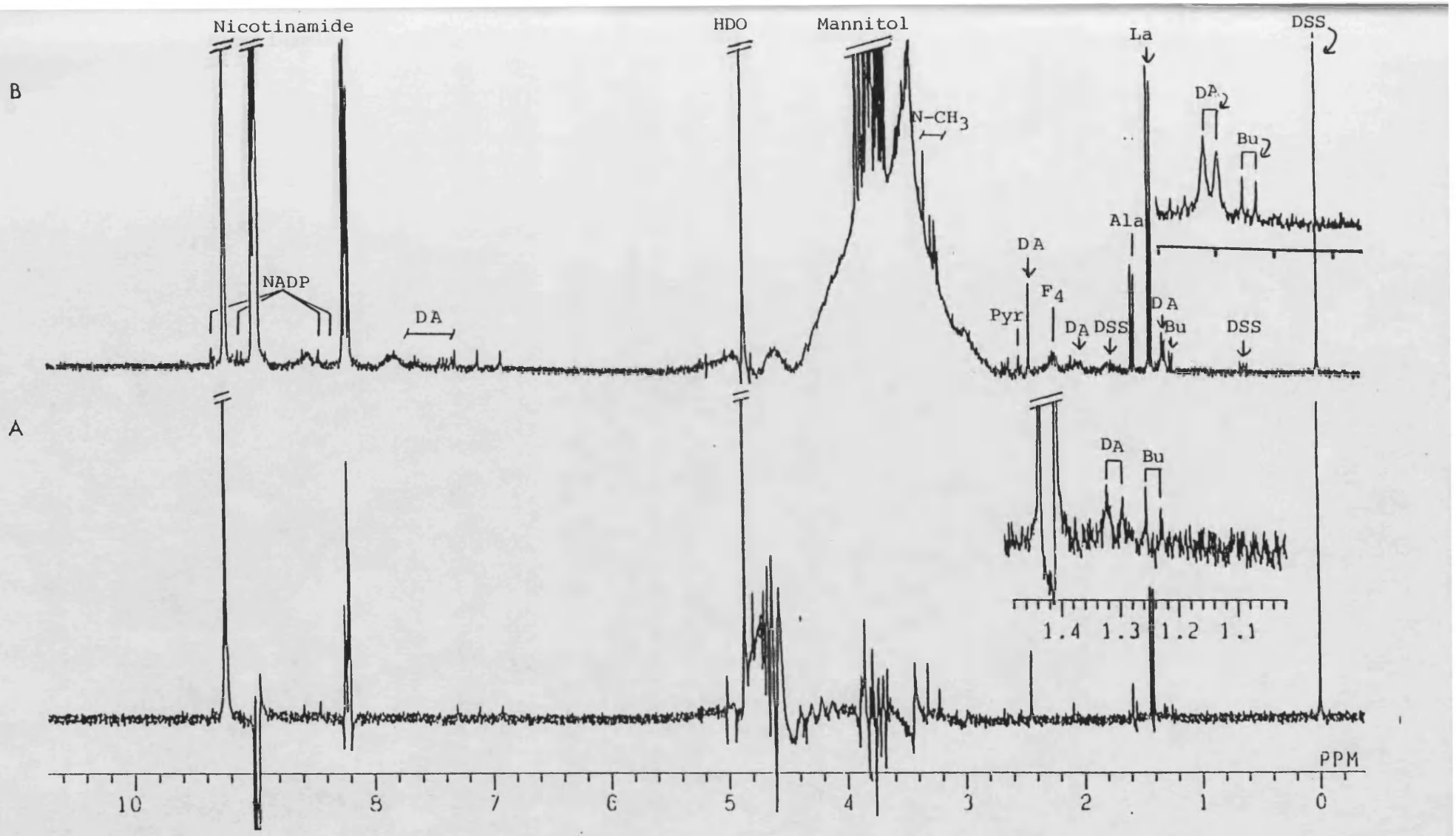


Fig. 4.4.2 270 MHz ^{1}H NMR spectra of acid extract of 3-methylcholanthrene treated 10,000 g liver fraction (contained 30 mg protein) after 30 min incubation with 2 mM daunorubicin (DA) (A) a spin echo spectrum (τ = 60 ms); (B) single pulse NMR spectrum. Resonances are labelled for DA and its metabolite, DA in addition to those seen in fig. 4.2.2. Note that DA and DA_3 resonances (about 1.04-1.35 ppm) are suppressed due to phase modulation and relaxation effects. Inset: expansions of methyl region, 1.0-1.5 ppm.

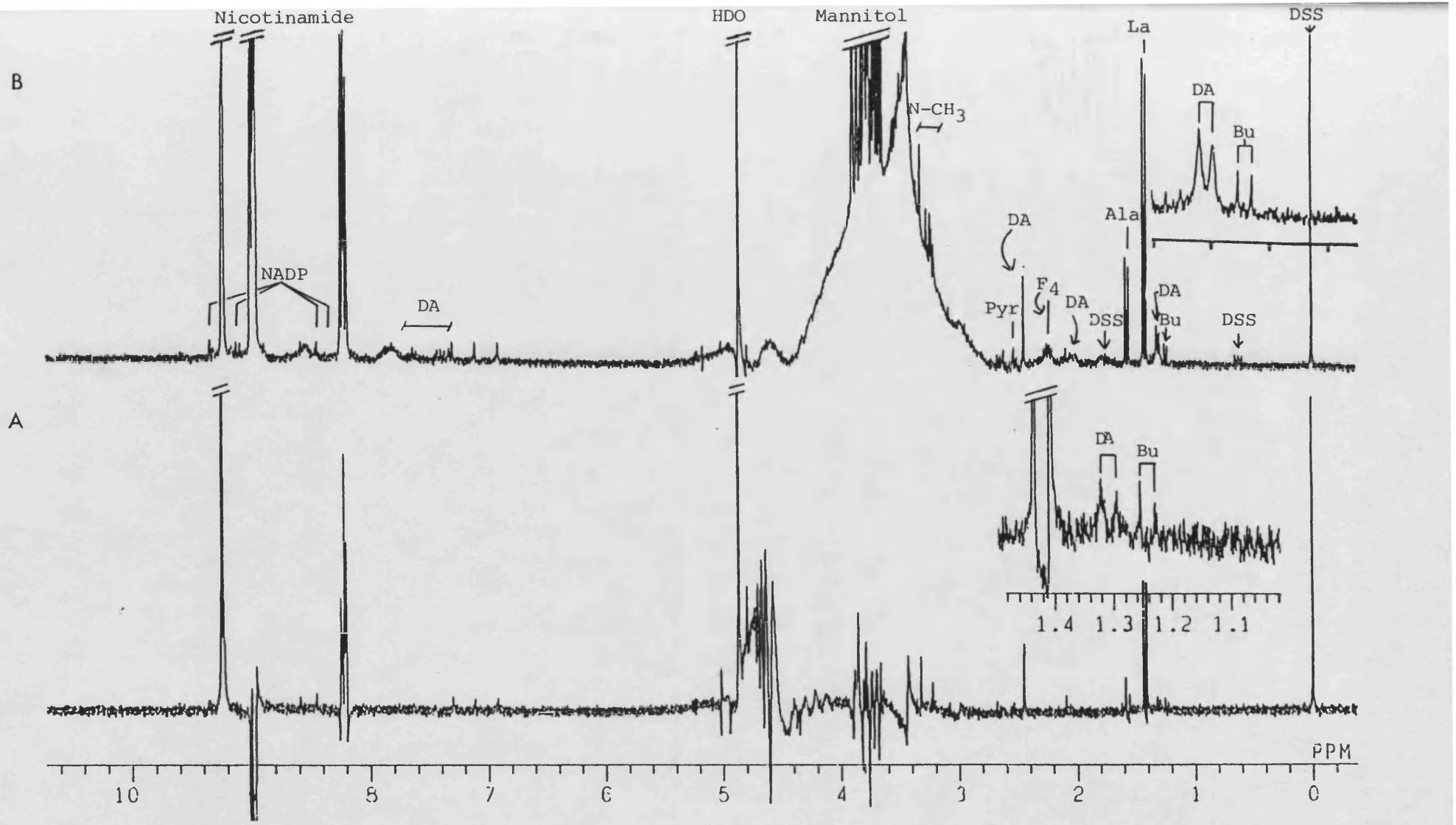


Fig. 4.4.3 270 MHz ^{1}H NMR spectra of acid extract of corn-oil treated 10,000 g liver fraction (contained 30 mg protein) after 30 min incubation with 2 mM daunorubicin (DA) (A) a spin echo spectrum (τ = 60 ms); (B) single pulse NMR spectrum. Resonance is labelled for DA in addition to those seen in fig. 4.2.2. Note that DA resonance (δ = 1.32 ppm) is suppressed due to phase modulation and relaxation effects. Inset: expansions of methyl region, 1.0-1.5 ppm.

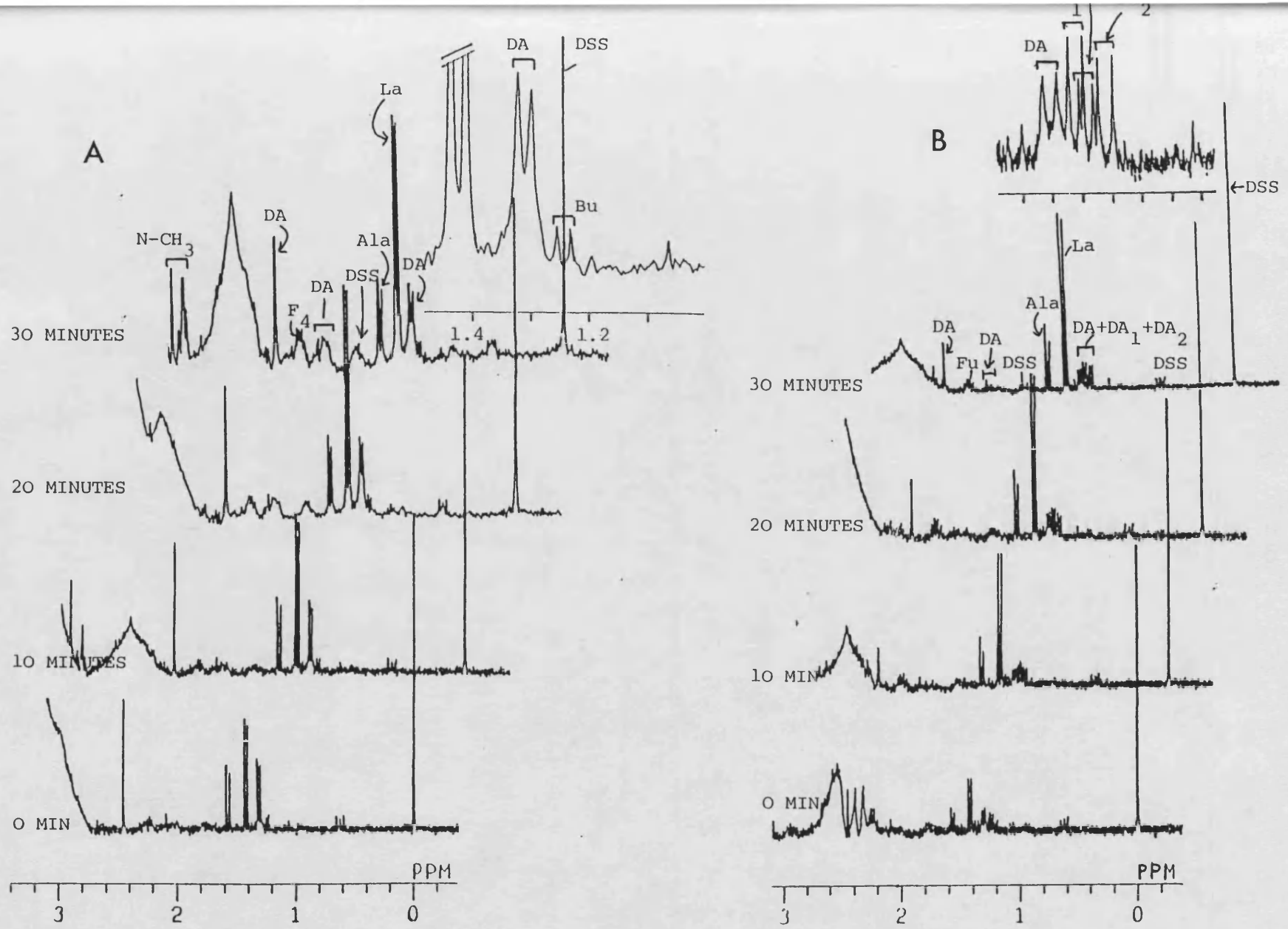


Fig. 4.4.4. Stacked plots showing aliphatic regions of the 270 MHz ^{1}H NMR spectra of acid extract of (A) saline, and (B) Phenobarbitone treated 10,000 g liver fraction (each contained 30 mg protein) after incubation with 2 mM daunorubicin (DA) at 0, 10, 20 and 30 min. Inset: expansions of methyl region, 1.1-1.4 ppm. A small reduction of DA intensity is found in A and a large one in B, along with the appearance of DA metabolites, DA_1 (δ = 1.25 and 1.28 ppm) and DA_2 (δ = 1.20 and 1.23 ppm).

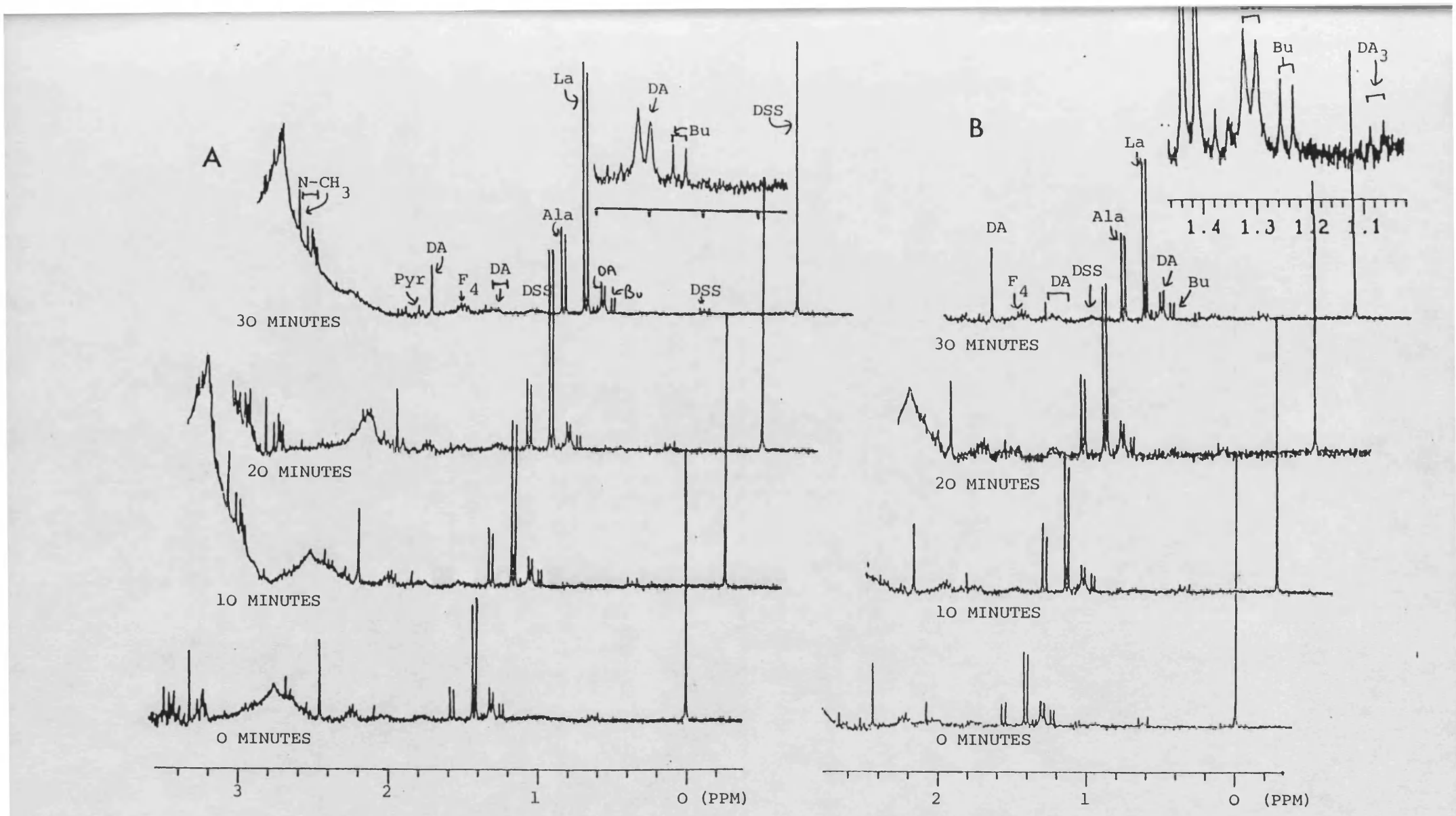


Fig. 4.4.5 Stacked plots showing the aliphatic regions of 270 MHz ^{1}H NMR spectra of acid extract of (A) corn-oil, and (B) 3-methylcholanthrene treated 10,000 g liver fraction (each contained 30 mg protein) after incubation with 2 mM Daunorubicin (DA) at 0, 10, 20 and 30 min. Inset: expansions of methyl region, 1.1-1.4 ppm. A small reduction of DA intensity is found in each A and B, along with the appearance of small intensity of DA metabolite, DA_3 (δ = 1.06 and 1.09 ppm).

CHAPTER FIVE

APPLICATION OF PROTON NUCLEAR MAGNETIC RESONANCE IN DRUG METABOLISM STUDIES

DISCUSSION

The application of ^{1}H NMR in drug metabolism studies has been investigated and will be discussed below. Assessment of NMR in quantitative studies has been undertaken and compared to spectrophotometry (SPEC) and HPLC analysis of urinary excretion of aspirin and paracetamol. The use of quantitative NMR for different drugs in body fluids has also been investigated using acemetacin in urine and D-penicillamine in plasma. Alternatively, in vitro metabolism and enzyme induction were studied by NMR using aminopyrine and daunorubicin as substrates.

5.1 QUANTITATIVE STUDIES BY ^{1}H NMR

5.1.1 COMPARATIVE STUDIES OF ASPIRIN

The major urinary excretion products of aspirin, i.e. salicylic acid (SA), salicyluric acid (SUA) and gentisic acid (GA) were quantified by SPEC and NMR and compared to analysis by HPLC. The aromatic resonances of SA, SUA and GA, and N-acetyl methylene resonance of SUA could be detected in 400 MHz ^{1}H NMR spectra (fig. 3.1.1 and tab. 3.1.2) of intact urine from a patient suffering from aspirin overdose, but were too extensively overlapped with endogenous compounds (hippurate resonances at 4 ppm, 7.5-7.9 ppm and indoxyl sulphate resonances at 7.2-7.7 ppm) for quantification to be attempted. It is easier to quantify a methyl signal (three magnetically equivalent protons) than the single proton of doublet, triplet or multiplet as are often the case in the aromatic region,

providing interfering compounds are absent. In the present study the problem arose from the overlapping signals of aspirin metabolites (even at high field) which meant that quantification of aspirin metabolites by NMR was abandoned leaving SPEC and HPLC methods for quantitative analysis.

The present study has shown significantly lower ($P<0.05-P<0.01$) amounts of SA in 3 out of the 4 patients studied compared to the HPLC results (see table 3.1.7) using Student's t-test. In contrast, the SPEC results of SUA and GA in all patients were significantly larger ($P<0.05-P<0.002$) than those obtained by HPLC (see section 3.1.8-3.1.9). The lower SA values by SPEC may be the result of incomplete extraction or the loss of sample during extraction prior to the determination of electronic absorption which is based on a purple colour given by the salicylate ion with ferric salts in weakly acidic solutions[34,235]. The larger SPEC results may be due to interference from other compounds(s) extracted with SUA (giving a purple complex with ferric salts) or with GA (giving a green complex with the Folin-Ciocalteau reagent)[34].

In the case of moderate aspirin poisoning treated with glycine (orally), the percentage of glycine conjugate of SA excreted in the urine as SUA was found to be lower (45-54% of the total salicylate excreted, table 3.1.3-3.1.6) than that after a therapeutic dose. Thus, after aspirin overdose, urinary excretion of SA, GA, SPG and SAG was increased and these metabolites therefore make a greater contribution towards overall salicylate elimination (fig. 3.1.3-3.1.4). In contrast, in a patient (A4) with severe poisoning (treated with bicarbonate for urine alkalinisation), SA was the major urinary metabolite (45.1%) with only 28.9% of the excreted

product being SUA. Urinary SA was higher in a patient treated with urine alkalinisation compared to those treated with glycine. This was attributed to the higher urine pH (above pH 7.0) in the former, which caused an increase in renal clearance of SA[239,240]. The net effect was a decrease in unionised SA entering the systemic circulation by passive tubular reabsorption for further metabolism, as is reflected by reduced fractional excretion of other metabolites (SUA, GA, SPG and SAG). The reduced urinary excretion of SUA demonstrated in the present work suggests that SA formation and/or elimination may be capacity limited. Following an intravenous[241] or oral[242] administration of SUA, it was found that SUA elimination was fast and not rate-limited. Published work suggests that the rate-limiting step after aspirin overdose is SUA formation rather than renal elimination[241,242].

5.1.2 COMPARATIVE STUDIES OF PARACETAMOL

In the present study ^1H NMR has been used to investigate the urinary excretion of paracetamol (P) and its metabolites paracetamol glucuronide (PG) and paracetamol sulphate (PS) in P overdose patients. ^1H NMR quantitative data has been compared to that obtained from HPLC and SPEC.

Typical 400 MHz ^1H NMR spectra of urine from patients suffering from P overdose (fig. 3.2.1 and 3.2.2) are shown. P and its metabolites as well as various endogenous metabolites were readily detected, each with characteristic shifts, intensity ratios and sometimes spin-spin coupling patterns (table 3.2.1 and 3.1.1 for P metabolites and endogenous metabolites respectively). The large signal of H_2O in urine samples was adequately overcome by applying homogated secondary irradiation at the H_2O resonance (δ =

4.8 ppm). In the present case, the N-acetyl methyl signals of P and its metabolites gave sharp peaks which could be quantified as this signal was in a region of the urine NMR spectrum where there were few signals from endogenous metabolites. A problem arose from the partially overlapping peaks in the narrow range of resonances (2.13-2.17 ppm) of the N-acetyl groups of the metabolites. However, these signals could be well-resolved using high field strength spectrometers, i.e. at 400 MHz, where their signals were better dispersed. Moreover, the resolution was enhanced by application of a Gaussian function to the FID before Fourier transformation, with careful attention to phase correction.

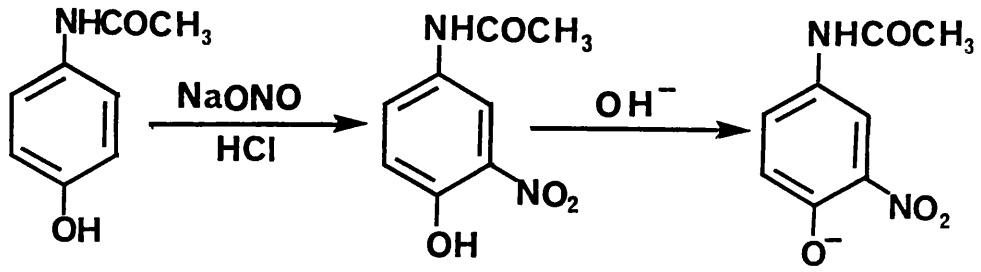
^{1}H NMR spectra of P and its metabolites have other characteristic resonances in addition to the N-acetyl methyl resonances described above, i.e. the aromatic ring protons which gave certain coupling patterns and other resonances in the aliphatic region (table 3.1.18). Examination of the urinary metabolic profile of P after overdose by ^{1}H NMR revealed PG as the major metabolite (59-73%) followed by PS (18-30%) in total urine collected over 13-48 h (unknown in 2 patients) based on the total recovery of P, PG, PS and PMA, in P overdose. From table 3.1.12, PG / PS was initially found to be 2.0 to 3.5 and as well as the (PG+PS)/ PMA ratios were found to decrease with time, in all patients after treatment with methionine orally or N-acetylcysteine intravenously.

In the present study, the acetyl methyl peaks of P, PG and PS in NMR spectra have been successfully quantified and gave reliable results compared to those of HPLC. Paracetamol cysteine (PC) was detected, but not quantified due to its extensive overlap with paracetamol mercapturic acid (PMA) and methoxy paracetamol.

However, PMA could be quantified easily from its side-chain N-acetyl methyl resonance at 1.86 ppm without interference from other metabolites. Another possibility of interference is the resonances of methionine, the drug administered for P overdose treatment, which gives signals at 2.12 ppm for the methyl and 2.16 ppm for the methylene groups. However, free methionine signals were either not detected or present in very low concentration in the urine sample studied.

The NMR results of mean concentrations of urinary P, PG and PS in intervals after P overdose were statistically similar ($P>0.05$) to those of HPLC (table 3.2.9-3.2.11). However, quantification by NMR appeared to give more variable results with SD up to 8%, whereas HPLC gave SD value less than 1%. The major problem that arises from NMR quantification is sensitivity. NMR can quantify the metabolite down to 0.1 mM, whilst HPLC gives results in uM range. However, the high concentrations of metabolites in overdose gave no problem in quantitative analysis by NMR.

In the case of the SPEC method, only PG determinations gave reliable results (i.e. no significant difference) compared to those by HPLC. P and PS concentrations gave significantly higher values than those by HPLC ($P<0.05$). The common SPEC method for P determination proposed by Chafetz *et al.*[236] was reported to give no interference from structurally similar drugs such as phenacetin and p-aminophenol. Paracetamol requires preliminary nitrosation with nitrous acid to form 2-nitro-acetamidophenol which can be measured by its colour in alkaline solution, as follows:



λ_{max} = 250; 285 nm λ_{max} = 370 nm λ_{max} = 430 nm

The SPEC results obtained could indicate that acid- or alkaline-labile metabolites(s) present in urine were converted to P in the method used giving a higher result. PG and PS were measured indirectly by converting them to P with enzymes, β-glucuronidase for PG, and sulphatase and β-glucuronidase for PG and PS. In the case of PS, another metabolite(s) may be "converted" by sulphatase which is subsequently detected by SPEC giving a higher value than that obtained by HPLC. Pretreatment of P metabolites was also required for HPLC determination, however, the separation process prior to detection by HPLC is considered to give more reliable results.

The metabolic mechanism of the toxicity of P has already been described (section 1.4.2.2). In a minor pathway accounting for about 5% of the dose, P is converted to a reactive intermediate, thought to be N-acetyl-p-benzoquinone imine[243], which combines with hepatic glutathione prior to urinary excretion[178]. After overdose,

however, the hepatic glutathione may be depleted, thus liver damage may occur as a result of covalent binding of the reactive intermediate with cellular macromolecules.

As described above, ^{1}H NMR spectra can be used to monitor drug metabolites and endogenous metabolites concomitantly. Therefore any alteration from normal or therapeutic conditions can be detected. The decrease of the ratio of PG/PS (table 3.2.12) after methionine and N-acetylcysteine treatment may suggest the increasing availability of sulphate, therefore aiding P elimination and hence reduce the amount of the oxidised intermediate. Even though paracetamol glutathione was not detected in the ^{1}H NMR spectra, its subsequent metabolic products, paracetamol cysteine (PC) and paracetamol mercapturic acid (PMA) were observed. It is thought that cysteine is obtained from methionine through homocysteine and cystathionine, or by straight conversion from N-acetylcysteine. The increasing pyruvate concentration observed in the spectra can be explained by the formation of its partial oxidative degradation to pyruvate and subsequent reduction to lactate by lactate dehydrogenases (fig. 5.1.1). Hence the lactate and pyruvate resonances were increased. In one patient, the disappearance of L-alanine was observed, suggesting a raised serum transaminase activity due to liver damage, and catalysis of L-alanine to pyruvate.

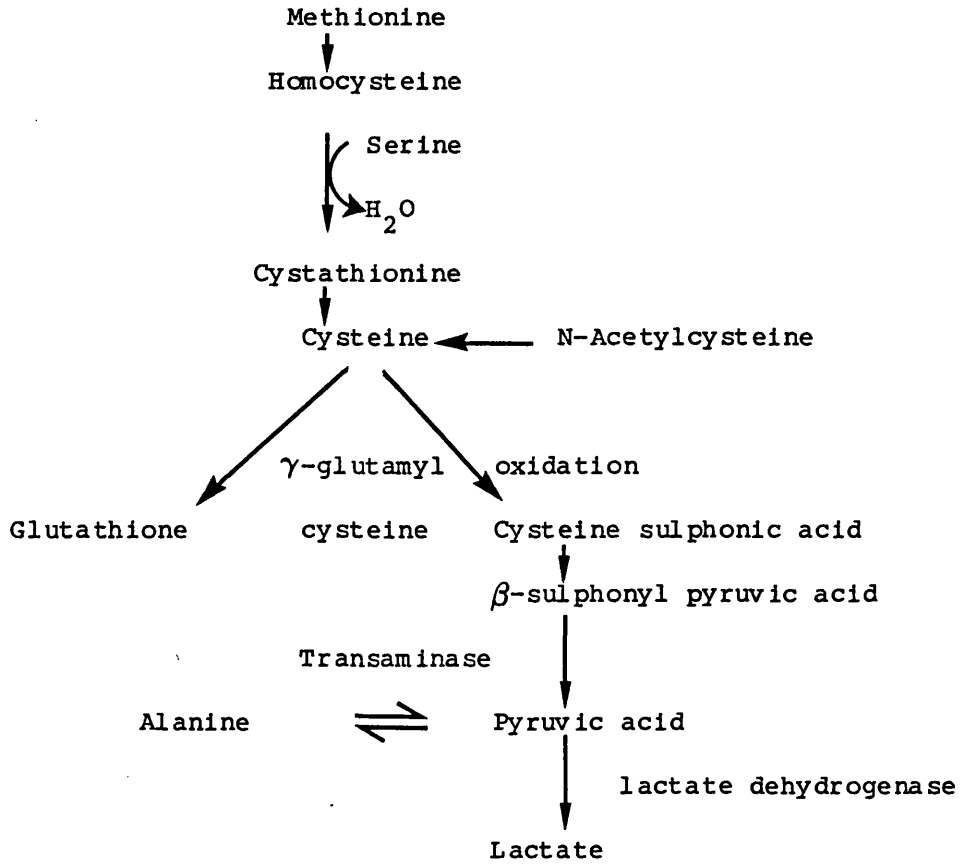


Fig. 5.1.1 Possible fate of amino acids administered and endogenous amino acids in cases of paracetamol poisoning

5.1.3 ACEMETACIN

Section 3.1 and 3.2 described the 270 MHz ^{1}H NMR spectra of endogenous compounds and drug metabolites (aspirin and paracetamol) in untreated urine samples with the aid of a simple solvent suppression method. However, free acemetacin (ACE) and its hydrolysis product, indomethacin (IN), gave resonances which were either very weak or not detected in unprocessed urine samples from 3 volunteers who took therapeutic doses of acemetacin. In order to increase the metabolite concentrations necessary for quantitative analysis, the urine samples were freeze dried and redissolved in

acetone-d_6 and ^2H_2O (3:2 v/v) to give 20-25 times the original concentrations. Despite the appearance of the acetone methyl resonance (2.2 ppm), this simple procedure also overcame the dynamic range problem caused by the strong water signal. However, since the signal-to-noise ratio of endogenous urinary metabolites was simultaneously improved, there were fewer spectral regions which provided little or no background interference. The loss of volatile components and the deuteration of acidic or exchangeable protons were not relevant in this case, thus allowing this method to be applied.

ACE and IN gave some resonances (table 3.3.1), the methyl resonances at 2.29 ppm and 2.31 ppm respectively showing the least interference from endogenous metabolites (fig. 3.3.1). Quantification of ACE and IN from 270 MHz ^1H NMR spectra was based on measurements of peak area for these singlet methyl resonances which were obtained by cutting and weighing paper traces. This technique allows accurate results in situations where the linewidths of the peaks are varied and the peaks of interest are slightly or partially overlapped (with acetoacetate signals, fig. 3.3.1), particularly when relatively insensitive low to medium field spectrometers are being used. The addition of some standard solution to 4 blank urine samples, freeze drying and redissolving in acetone-d_6 and ^2H_2O (section 2.3) showed that the analytical recovery of IN was good ($92.0\pm5.7\%$, n=4). Table 3.3.2 shows concentrations of urinary IN and very low concentrations of urinary ACE (not measured in some cases), suggesting that most ACE administered was converted to IN in the body.

270 MHz ^1H NMR spectra of freeze dried urine samples from a subject who had taken ACE (fig. 3.3.1) showed more endogenous

metabolites compared to those of intact urine (fig. 3.1.1, table 3.1.1) which were taken to be ACE metabolites. However, lack of standard metabolites available did not permit confirmation of these signals as ACE metabolites. The urine spectra of 1 subject showed a decrease in urinary glycine on the 1st day and in another volunteer no glycine was detected on the 8th day. This finding suggests that the pathway of metabolism of ACE involves conjugation with glycine in the liver, possibly depleting this endogenous substrate. Several other endogenous metabolites were monitored and found to vary. This is in agreement with previous HPLC findings which indicated that higher concentrations of glycine conjugated metabolite were excreted in volunteers who had taken ACE than those who had taken IN[182].

5.1.4 D-PENICILLAMINE METABOLITES

^{1}H NMR spectra of whole blood have been previously shown to contain resonances from both erythrocytes and plasma[117,89]. However, the spectra from whole blood were not well-resolved and reproducible. Spin-echo ^{1}H NMR spectra from resuspended red cells have also been observed[62]. Well-resolved spectra were generally obtained although the spectra sometimes contained only H_2O or broad unresolved resonances[111]. This problem was thought to be due to small amounts of paramagnetic deoxyhaemoglobin in the venous blood and also to erythrocyte sedimentation and movement during the course of spectral accumulation. NMR spectra from plasma alone were more highly resolved and reproducible[111]. Hence, in the present study plasma was chosen for the study of various endogenous metabolites as well as drug metabolites (table 3.4.1 and 3.4.2 for endogenous metabolites and D-penicillamine metabolites respectively). EDTA stabilised the D-penicillamine (PSH) in the

reduced form at room temperature or 4°C by complexing trace metals which catalysed thiol oxidation[244], and hence EDTA plasma was used to examine PSH and its metabolites.

270 MHz single pulse ^1H NMR spectra of EDTA plasma using secondary irradiation of the water signal (fig. 3.4.1) was hampered by the large number of overlapping resonances from macromolecules such as proteins and fats. However, in the spin echo technique broad resonances from macromolecules and relatively immobile groups disappeared via spin-spin (T_2) relaxation during the waiting periods (τ) of the two pulse sequence (see section 1.3.3.4). Sufficient time (2τ = 120 ms) then elapsed for the net magnetization associated with most broad resonances (short T_2) to decay to zero and thus not contribute to the spectrum. A large number of well-resolved signals from mobile protons of low molecular weight metabolites in plasma EDTA have been assigned (table 3.4.1). However, in quantitative studies the intensities of resonances have to be interpreted with caution since they may be influenced by both T_1 and T_2 relaxation. All spectra were accumulated with similar pulse repetition rates so that T_1 effects were likely to be similar in each case. The factors affecting T_2 include the binding of small molecules to macromolecules, to paramagnetic ions (e.g. Cu^{2+}) and viscosity differences between plasma samples. A study of creatinine concentration in plasma by spin-echo ^1H NMR[245] has shown only 70% recovery of this compound. In contrast, creatinine concentrations measured by ^1H NMR and the Jaffé method in urine where protein concentrations are negligible, have been reported to be in good agreement[94]. Thus, although spin echo technique was carried out to minimize the interference from macromolecules as well as to provide an aid to the assignment, some

factors remained that interfered with analysis by ^{1}H NMR, especially quantitatively. Therefore, in the present study an attempt was made to measure penicillamine (PSH) and its metabolites in the non-protein fraction of plasma after acid extraction. For comparison, NMR spectra of serum and heparin plasma ultra-filtration (fig. 3.4.2 and 3.4.3 respectively) were recorded and showed similar resonances as those of EDTA plasma except for the resonances of EDTA and trace metal complexes.

Several investigators have reported measuring plasma concentrations of PSH by spectrophotometry, chromatography or radioimmunoassay (see section 1.4.2.4). The occurrance of side effects in RA patients treated with PSH and the variation in efficacy seen with the drug might be related to plasma levels of the drug and its metabolites (1-10 mg/l)[246]. Technical difficulties in developing assays to routinely measure the drug in biological fluids have led to a lack of information regarding this situation. In the present study, however, PSH and its metabolites in the non-protein fraction of plasma EDTA were not detected in the spectra (fig. 3.4.2), although the plasma samples had been lyophilized and redissolved in $^{2}H_2O$ to obtain a 40 times concentration. Resonances of PSH and its metabolites (0.3 mM for each concentration) spiked in plasma-EDTA as described in section 2.10.1.3 (table 3.4.2) were obtained. This fact suggests that the non-appearance of PSH and its metabolites in the pooled plasma spectra from RA patients were due to the low concentration of the drug present in plasma. In addition, as in the case of acemetacin, lyophilized samples used to increase drug concentration also increased the signal-to-noise ratio of plasma components thus aggrevating the problem of "chemical noise" in drug measurement.

However, the appearance of low molecular weight endogenous compounds in intact and acid extract plasma (listed in table 3.4.1) should be of value in clinical and biochemical analysis.

5.2 IN VITRO METABOLISM STUDIES OF AMINOPYRINE AND DAUNORUBICIN BY ^{1}H NMR SPECTROSCOPY

5.2.1 GENERAL

In vitro metabolism of aminopyrine (DMAP) and daunorubicin (DA) was studied using ^{1}H NMR spectroscopy. Rat livers used were gently homogenised in an isotonic phosphate buffer, a process that ruptures the plasma membrane but leaves most of the internal organelles intact. The liver homogenates contain the subcellular organelles, e.g. nuclei, mitrochrondria and endoplasmic reticulum, which differ in size and specific gravity and thus sediment at different rates following differential centrifugation. Centrifugation at 10,000 g for 10 min sediments the nuclei, mitochondria and debris, leaving the endoplasmic reticulum, cytosol and soluble portion of the cell cytoplasm in the supernatant fraction. The subcellular organelles catalyse different metabolic reactions[247]. For example, the cytosol contains substrates and enzymes needed in glycolysis, gluconeogenesis, the phosphogluconate pathway, activation of amino acids and fatty acid synthesis. The cytochrome oxidase enzymes are located in the endoplasmic reticulum, whereas the enzymes of the citric acid cycle, electron transport and those concerned in conservation of oxidative energy in the form of ATP are located in the mitochrondria.

The 10,000 g liver fraction of the rat, which contained both the microsomal and soluble fractions, was used in the present study to compare the metabolism of DMAP or DA after enzyme induction by

phenobarbitone (PB) or 3-methylcholanthrene (3MC). Microsomal fractions (without the soluble fraction), which contained the cytochromes (obtained from the 10,000 g liver fraction after centrifugation at 40,000 g for 60 min), were also considered for use. The disadvantage of using only the microsomal fraction for *in vitro* metabolism was that little or no metabolites were observed by ^{1}H NMR spectroscopy due to low metabolic activity leading to low concentrations of metabolites. The low metabolic activity of the 40,000 g pellet (i.e. microsomes) could be attributed to the loss of microsomal enzymes in the isolation of the microsomal fraction and possibly due to the lack of cofactors. *In vitro* metabolism of DMAP and DA was more successful using the 10,000 g fraction and therefore this fraction was used routinely in the study despite the fact that phase II conjugation reactions might also occur and complicate the ^{1}H NMR spectra.

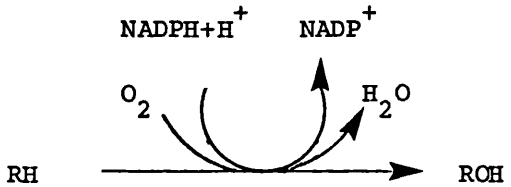
The supernatant fraction used contained glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PDH) which in the presence of nicotinamide adenine dinucleotide phosphate (NADP) generate the reduced form, NADPH, required for drug metabolism according to the following reaction:

$$\text{Glucose-6-Phosphate} + \text{NADP}^+ \underset{\text{Mg}^{2+},\ \text{G6PDH}}{\rightleftharpoons} \text{6-Phosphogluconolactone} + \text{NADPH}+\text{H}^+$$

When the 10,000 g liver fraction is used as the drug metabolising enzyme preparation, there is no need to add G6PD since it is present in this fraction. It can be seen from the above reaction that G6P, NADP and Mg^{2+} need to be added as cofactors. G6P must be added to the enzyme preparation since livers from starved rats for 24 h had low concentration of G6P due to increased

glucose-6-phosphatase and decreased glucokinase activities[248]. Mg^{2+} must also be added to *in vitro* preparations since it forms complexes with the phosphate groups of the glycolytic intermediates, e.g. G6P, which provide the binding sites for many of the glycolytic enzymes[224]. Also, in order to prevent destruction of the pyridine nucleotide (part of NADP) by tissue nucleosidases, especially in a long incubation time, nicotinamide was added to the *in vitro* incubation mixture.

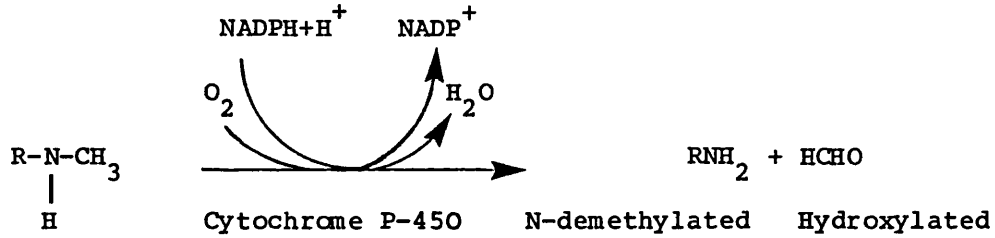
Monooxygenase enzymes (cytochromes P-450/P-448) in the microsomal fraction carry out specialised oxidative reactions, e.g. hydroxylation and carboxylation, in which one of two oxygen atoms is incorporated into the main substrate, the other being reduced to H_2O with two H atoms donated by the cosubstrate. Since they oxidise to different substrates simultaneously, they are also called mixed-function oxidases. In the present study, NADPH was used as the cosubstrate. The most numerous and complex monoxygenation reactions are those employing the cytochrome P-450 family of haem proteins. The general reaction equation for monoxygenases can be expressed for the hydroxylation reaction as shown below:



Hydroxylated product

In the case of DMAP, the above reaction can be modified for

N-demethylation as follows :



For DA, the metabolism pathways in the rat liver is shown in fig. 1.5.2. The above figures show the simplified outline of the action of cytochrome P-450, which has many complex intermediate steps. Cytochrome P-450, which participates in enzymatic monoxygenases, accepts two electrons from NADPH and introduces one oxygen atom into the substrate.

In the present study, livers were obtained from rats pre-treated with PB, 3MC, saline (control for PB), or corn-oil (control for 3MC). PB and 3MC differ in the course and intensity of cytochrome induction. The microsomal portion obtained from control livers contained cytochrome P-450 which could be characterized from the carbon monoxide (CO) complex of its reduced form which absorbs light maximally at 450 nm (see fig. 4.1.1). The CO complex of reduced cytochrome after PB induction exhibited an increased absorption compared to those of controls at 450 nm (see table 4.1.1). Thus cytochrome P-450 concentration had been induced after PB treatment (see section 4.1). After 3MC induction, the peak absorbance of the CO reduced cytochrome complex had been shifted from 450 to 448 nm, indicating the presence of cytochrome P-448. The concentration of cytochrome as an enzyme protein can be related to the protein concentration in the microsomal and soluble fraction to indicate the extent of the induction.

It is now well known that there are various forms of microsomal mono-oxygenases with different substrate specificities and inducibilities, but the 'P-450' family of cytochromes plays a large role in drug metabolism. A detailed study of the interaction of cytochrome P-450 with various substrates is needed to understand the mechanism of catalysis of this protein. This interaction is usually studied by optical methods[249,250] which gives no idea of the spatial structure of the enzyme-substrate complexes. NMR has been used to study the relationship between drug or substrate binding and its oxidation by the two cytochromes P-450 or P-448 induced by PB or 3MC respectively[215,216] (see section 1.5.1). A number of similar studies have been performed by ^{1}H NMR longitudinal relaxation rate measurements. Even though the binding studies give more detailed information, they cannot be readily adapted to study increased activity of drug metabolising enzymes induced by foreign compounds or the metabolic pathways of drugs. Enzyme induction increases the activities of drug metabolising enzymes which leads to an accelerated biotransformation of drugs *in vivo*[248]. The activity of all enzymes will have some influence on the rate of the pathway, but some will be much more important than others.

An ^{1}H NMR method has been presented for the determination of the effect of compounds that stimulate the biosynthesis of liver microsomal drug metabolising enzymes, cytochrome P-448 and/or P-450 and their activities towards two substrates, aminopyrine (DMAP) and daunorubicin (DA). ^{1}H NMR spectra were obtained from PB, 3MC, saline or corn-oil pretreatments with or without drug substrates added. ^{1}H NMR spectra can be obscured by the presence of certain endogenous macromolecules such as fats. For this reason, rats were

starved for 24 h before being killed. During starvation, the whole direction of intermediary metabolism is switched to the net production of glucose by glycogenolysis, and, as fasting continues, by gluconeogenesis from pyruvate, lactate, glycerol and amino acids. During starvation, the energy requirements of the liver are supplied by the oxidation of fatty acids. Thus, the fatty acids which would give problems in centrifugation and in the determination of protein concentration, as well as broadening the ^{1}H NMR spectra, were eliminated.

Water suppressed ^{1}H NMR spectra of 10,000 g liver fraction are shown in fig. 4.2.1. The spin echo pulse sequence results in the elimination of many of the broad resonances in the normal spectrum due to protons of macromolecules which have short spin-spin relaxation times (T_2 values), such as proteins and unsaturated fatty acids[112,113,237,238]. In the single pulse spectrum, however, some sharper resonances were superimposed on the broad envelope of overlapping resonances. Some signals, at 0.9 and 1.3 ppm (F_1 and F_2), still remain (fig. 4.2.1) and can be assigned[237,238] to the terminal $-CH_3$ and $-(CH_2)_n-$ protons respectively of mobile fatty acids, probably part of triglycerides and phospholipids. Canioni et al.[251] attributed some sharp ^{13}C NMR signals from liver observed by topical magnetic resonances to fatty acids in phospholipids. Triglycerides account for about 9-30% of the total lipids in liver, and phospholipids are about 65%. The glucose resonances, in the region 3.40-3.95 ppm, are broadened in the single pulse experiment and are phase modulated in the spin echo experiment (fig. 4.2.1A and B). Three sharp singlet resonances appeared in the region 3.1-3.3 ppm. According to Birdsall et al.[237] and Frost and Gunston[238] the ^{1}H NMR spectra of [$^{2}H_4$]methanol extracts of

lyophilized liver cells contained intense characteristic resonances for the phosphatidylcholine head group and fatty acyl chains of phospholipids. This suggests that the singlet resonances observed around 3.2 ppm in intact cells of liver are also from choline head groups. Other possibilities of resonances in this region are betaine and carnitine[252]. In the intact microsomal and supernatant sample, no peaks appeared in spectra corresponding to DSS added as the internal reference and standard. The disappearance of DSS might be due either to localisation within small intracellular compartments or to binding to macromolecules. A sharp doublet assignment for lactate (δ= 1.40 ppm) appeared in the single pulse spectrum and phase modulated in spin echo spectrum.

In the intact microsomal and supernatant spectra, problems arose from the broadened resonances in the single pulse experiment and the phase modulated resonances in the spin echo experiment which would not permit quantitative measurement. To overcome these problems, the reconstituted samples were extracted by trichloroacetic acid (TCAA) after set incubation times to disrupt cells, and denature and precipitate proteins. From fig. 4.2.2, it can be seen that the spectra of acid extracted microsomal and supernatant fraction contain more well resolved resonances for cellular metabolites. The single pulse spectrum (fig. 4.2.2.B) includes resonances for amino acid (L-alanine), ketone body (β-hydroxybutyrate), acetate, lactate, pyruvate, glucose and the internal standard DSS. Signals for the mobile fats observed in the non-extracted spectra were notably absent in the spectra of the acid extracted sample, except that of F_4. Such fats may precipitate with protein and cell membranes and be removed by centrifugation. The spin echo ^{1}H NMR spectrum of the acid extract

was essentially the same as those of the non-extracted sample except for shifts due to pH differences between the samples. The N-methyl resonances, shifted to 3.2-3.4 ppm, were now better resolved. However, there was a loss of some signals in this region, further confirming that the resonances at this position were due to the choline head group of a phospholipid. The broad envelope resonances of protein disappeared in the region of 1.0-3.4 ppm, but the broad signal due to macromolecules still existed at 3.4-4.4 ppm. Although these broad signals were eliminated in the spin echo spectra, there was a decrease in singlet resonance intensities and the some doublet resonances intensities observed at low concentration disappeared. These results suggest that the use of spin echo experiments quantitatively or for the monitoring of low concentrations of sample components is not advisable.

The single pulse spectra of the acid extract of the 10,000 g liver fraction gave more resolved resonances for drugs and their metabolites (discussed below) and some endogenous components, in the region between 1.0-3.4 ppm. The extraction with TCAA was also used to stop the <u>in vitro</u> metabolism at various time intervals due to its ability to inhibit enzyme activities by denaturation and precipitation (with no change of peak intensities in acid extract spectra after 24 h, 4^{o}C). Spectra of acid extracted 10,000 g liver fraction obtained from untreated, PB- or 3MC-treated rats were monitored using ^{1}H NMR spectroscopy. Over a 30 min incubation (see 2.10.2), glucose intensities were reduced by 0.6 to 0.8 fold with an increase in lactate and L-alanine resonances, this may be evidence for glycolysis occurring in the reconstituted enzyme system resulting in synthesis of pyruvate which in the presence of NADPH+H^{+} and lactate dehydrogenase, was converted subsequently to

lactate. However, the increase in lactate concentration in PB treated samples over 30 min incubation was greater (3.3 fold) than that found after 3MC, saline or corn-oil treated samples (1.2-1.7 fold). In the 10,000 g liver fraction there was an increase in L-alanine 2.1-2.4 fold over the 30 min incubation period indicating that pyruvate could also be converted to L-alanine through a transaminase enzyme. Endogenous metabolites such as pyruvate are produced and utilised very rapidly and hence may be the reason for the variable small amounts detected over the 30 min incubation. The concentration of a ketone body, β-hydroxybutyrate, which is the product of fatty acid oxidation in mitochondria, was found to be constant in all the ^{1}H NMR spectra. This fact suggested that the 10,000 g liver fractions were contaminated by β-hydroxybutyrate from mitochrondria in the homogenizing process. However, pyruvate and the high level of lactate found indicated that the 10,000 g liver fraction were separated from mitochondria since they are converted to acetyl CoA in the mitochondria and further oxidised through the citric acid cycle for energy production. Some of the enzymes were differentially induced by PB and 3MC as shown by the changes of endogenous lactate between the two treatments. The reconstituted enzyme systems appeared to be active for endogenous catalysis, some served in the catabolism (e.g. glycolysis), and some functioned in the synthesis of cellular metabolites (e.g. lactate and L-alanine).

5.2.2 AMINOPYRINE

The reconstituted enzyme systems above consisted of both microsomal and soluble fractions and added cofactors which were then incubated in the presence of DMAP or AP, as the model

substrates. In contrast to the control spectra, the resonances in the region expected for glucose seemed to be broadened and featureless, but increased in intensity in the presence of DMAP and its metabolites. This result is in agreement with that of an experiment performed by Nicholson et al.[252] when paracetamol was incubated with hepatocytes. They considered that the endogenous metabolite might be an intermediate in glycogenolysis needed for glucuronide formation, even though the ^{1}H NMR spectrum of a solution of glycogen in $^{2}H_2O$ was found to be broad and featureless. However, the increased intensities of lactate in 10,000 g liver fraction incubated with DMAP were found to be the same as those of control and may indicate glycolysis. The rate of increase of L-alanine resonance intensities were found to be lower than those of the control spectra (1.5-1.6 fold and 2.1-2.4 fold respectively), suggesting that DMAP inhibits the transamination reaction.

When DMAP was incubated with liver microsomal and soluble fractions at 37°C for 30 min, three new singlet resonances were observed at = 2.50, 3.34 and 3.35 ppm and aromatic resonances at 7.4-7.8 ppm by spin echo ^{1}H NMR. However only that at 2.50 ppm appeared in single pulse spectrum since the other two peaks were obscured by the broad envelope of macromolecules (δ = 3.4-4.8 ppm) and peak distortion by water irradiation. The intensity of the singlet resonance at 2.50 ppm due to the methyl signal of DMAP had decreased after 30 min incubation. However, no peaks appeared in the spectral region corresponding to DMAP metabolites (for metabolic pathways of DMAP, see fig.1.5.1). Characteristic, well resolved resonances for the metabolite, 4-aminoantipyrine (AP), were observed in the NMR spectrum when a standard addition of AP

was made to this suspension. This result is similar to that obtained by Nicholson et al.[252] with paracetamol. Paracetamol metabolites expected after 20 min incubation with hepatocyte cells were not observed in the NMR spectrum of the suspension. They suggested that the non-appearance of the metabolites was due to the weak concentrations which were difficult to observe in the presence of cells when the signal-to-noise ratios were low and was not due to adverse line broadening resulting from (extracellular) diffusion in inhomogenous fields created by the cell membranes. The problem in this study was overcome by acid (TCAA) extraction where the appearance of DMAP and its metabolites,4-monomethylaminoantipyrine (MMAP), AP and unknown compounds, could be observed and monitored (see fig. 4.3.2).

Cytochromes were found to be active in the reconstituted systems, the liver microsomal and soluble fractions with added cofactors as shown by DMAP N-demethylation (see fig. 4.3.2). Demethylation activity (see metabolic pathway of DMAP in fig. 1.5.1) was followed in 270 MHz ^{1}H NMR spectroscopy by the disappearance of DMAP and the formation of its metabolites, MMAP and AP. Over the 30 min in vitro incubation, DMAP, the initial substrate in the PB treated samples was metabolised rapidly. Its disappearance was significantly faster than in 3MC treated or control samples (see table 4.3.2 and fig. 4.3.3). The formation of the metabolite MMAP was significantly higher in the PB treated samples, although another metabolite, AP, formed at the same rate as it occurred in 3MC treated or control samples. These results revealed that cytochrome P-450 which plays a central role in DMAP metabolism in the liver microsomes from PB treated rats had been induced. It was found that PB increased the enzyme activity in

N-demethylation of the first step, i.e. DMAP to MMAP, but not that of the second step, MMAP to AP. The disappearance of substrate, DMAP, and the formation of the metabolites, MMAP and AP, in the 3MC treated samples were found to be the same as those of control samples. This suggests that the cytochrome P-448 induced by 3MC (see section 4.1) does not increase the metabolic rate of DMAP. Quantitative ^{1}H NMR analysis of DMAP at the beginning of the reaction revealed about 98% recovery (see table 4.3.1). The recovery after the reaction (at 4,10,20 and 30 min) of DMAP and its equivalent in metabolites was only about 75%. The appreciable amount of substrate disappearance (25%) found in the early stages of the reaction (see table 4.3.2), which could not be accounted for by metabolic products may be derived directly from the substrate through another pathway. In all the 10,000 g liver fractions that were incubated with DMAP, the substrate DMAP concentration was still decreasing after 20 min, but MMAP was produced with a higher rate than previously while the concentration of AP, a product of MMAP, was reasonably constant (see fig. 4.3.3). The unknown metabolite found in the PB treated samples at 60 and 120 min suggested alternative pathways arising from DMAP and MMAP, and not from AP since the unknown metabolite appearance was accompanied only by decrease of DMAP and MMAP. These results are in agreement with the metabolic pathways of DMAP described in section 1.5.2.1 (fig. 1.5.1).

5.2.3 DAUNORUBICIN

In the spectra of the samples where DA were incubated (fig. 4.4.1-4.4.3), it was impossible to monitor the resonances within the usual chemical shift range of sugars since the DA preparation

used contained mannitol as a stabilizer which resonated at 3.86-3.93 ppm. The lactate resonances appeared to be induced by DA or mannitol in the 3MC, saline or corn-oil treated samples (2.1-2.3 fold), but not in the PB treated one (3.4 fold) where the concentration was found to be the same as the PB treated control samples, presumably due to the maximal enzyme activity in the system. Mannitol is not absorbed after oral intake, but there may be a possibility that it is converted to a glycolytic intermediate in the in vitro incubation resulting in a higher concentration of lactate. In contrast to the samples for DMAP metabolism, DA or mannitol seemed to increase L-alanine resonance intensities, compared to those of control spectra (2.8-3.5 fold and 2.1-2.4 fold respectively).

Fig. 4.4.4A and B, and 4.4.5A and B give typical spectra for DA following incubation for 0,10,20 and 30 min. The concentration of DA was too small to be accurately quantified and the data obtained was largely qualitative. In the reconstituted system, consisting of the microsomal and soluble fractions and co-factors, the rate of DA metabolism was greatest in PB treated samples, followed by those of 3MC and control samples respectively, monitored by the disappearance of the substrate. DA metabolite resonances which appeared in PB treated samples (DA_2 and DA_3) were in different positions to the one from 3MC treated samples (DA_3). In the control samples, no metabolites were observed, presumably due to their low concentration. These results indicate that PB and 3MC induce different metabolic pathways of DA.

5.3 APPLICATION OF ^{1}H NMR SPECTROSCOPY TO DRUG METABOLISM STUDIES

^{1}H NMR spectroscopy has been used to detect a wide range of intermediary metabolites present in human biological fluids such as urine and plasma, and microsomal and soluble fractions of rat liver. ^{1}H NMR is also capable of providing full quantitative analysis of drugs, metabolites and endogenous substrates simultaneously. In the present study, the potential of ^{1}H NMR has been demonstrated for widespread application in drug metabolism studies and the following conclusions can be drawn :

5.3.1 URINE ANALYSIS

Urinary excretion of drugs and their metabolites plays a large role in providing information in drug metabolism studies. Using ^{1}H NMR, measurement of urinary excretion can usually be achieved without extensive physical preparation or chemical pretreatment if compounds are present in sufficiently high concentration. In the present study urine analysis by NMR was applied to aspirin, paracetamol and acemetacin. Successful analysis by NMR, compared to HPLC and spectrophotometry methods when available, was only obtained for paracetamol and its metabolites, and the acemetacin metabolite, indomethacin. The detection of metabolites in unprocessed urine samples is possible provided they have suitable resonances and are present in the mM concentration range. Reliable quantification of drug metabolites in urine samples requires the molecules to give signals in spectral regions low in "chemical noise" (see fig. 3.1.1). Wilson *et al.*[107] have stated that the spectral region of low chemical noise is where background interference from other molecules is small. This condition is most easily achieved in analysis of drugs which have large therapeutic

doses or in overdose when their concentrations are much larger than those of endogenous compounds. High field strength spectrometers (e.g. those operating at 400MHz) have been used in order to obtain maximal sensitivity and good dispersion of signals. This minimises overlap of drug metabolite resonances and those from the more abundant endogeneous urinary components. Moreover, the use of resolution enhancement techniques, e.g. application of a Gaussian function to the FID prior to Fourier transformation, can partially overcome the problem of reduced signal dispersion, especially at lower field strengths (e.g. 270 MHz). Quantification cannot be carried out when a compound does not have isolated resolved signals in its spectrum or if they overlap with endogeneous compounds, as proved the case for aspirin.

Apart from chemical noise, analysis using ^{1}H NMR has another disadvantage, i.e. insensitivity. Owing to the inherent insensitivity of NMR, higher concentrations of compounds are required than those for HPLC when ng or even pg quantities can be detected. However, the limitations of sensitivity by NMR (found to be approximately 0.1 mM) can be overcome by lyophilization, i.e. freeze drying and redissolving of urine samples to get higher concentrations. This result gives recoveries and reproducibility as good as those obtained from unprocessed samples, as found in the measurement of the acemetacin metabolite, indomethacin.

In the drug metabolism studies described above, NMR has been used to provide detailed metabolic information on both exogenous (drugs and their metabolites) and endogenous compounds concurrently, which may be of importance in assessing changes as a result of treatment or overdose. A major advantage of NMR is that it provides a "fingerprint" picture on a variety of metabolites

specifically, without the need to preselect the detection conditions provided the metabolites possess protons suitable for observation by NMR spectroscopy. In contrast, HPLC requires sample preparation and column separation to give only a single peak for each metabolite which is subsequently detected. In the case of SPEC, there is a need for sample pretreatment, such as extraction and/or chemical reaction(s) to give a species which absorbs the visible light selectively, giving only one peak for one metabolite in one time. This, however, is often difficult to achieve.

The typical "finger-prints" of endogenous metabolites can be substantially altered following drug therapy. For example, i) after paracetamol overdose, providing indication of liver damage (e.g. large concentration of lactate), ii) after treatment with methionine and/or Parvolex and iii) after acemetacin administration (see section 3.1, 3.2 and 5.1). Given the rapid, direct and simultaneous estimation of low molecular weight metabolites, it seems likely that, while NMR instrumentation is expensive the technique may be of value in clinical and biochemical investigations and particularly in toxicological studies.

5.3.2 PLASMA ANALYSIS

The ^{1}H NMR spectra of plasma (fig. 3.4.1) contains fewer resolved resonances than the spectra of urine. Macromolecules such as fats and protein which are present in large concentration in plasma, hamper quantitative analysis of drug metabolites. However, metabolic information from plasma samples may be available from NMR studies, but such measurements rely on the spectral simplication achieved by applying spin echo techniques, providing the compounds can be detected by NMR (see section 5.2). The spin echo method has

the advantage of phase modulation of multiplets as an aid to peak assignment. Accurate quantification of peak areas in spin echo spectra is complicated by the effects of molecular diffusion (if there is B_o field inhomogenity), T_2 decay or binding with macromolecules. This problem can be overcome by extracting macromolecules from plasma, e.g. by protein denaturation or 1000 molecular weight cut off ultrafiltration. Using these techniques, more resolved spectra can be obtained and quantitative analysis could be attempted providing a suitable resonance is present in the NMR concentration range (as in urine analysis). Despite its detection in a non-protein plasma fraction after spiking control plasma with standard compounds (see section 2.10.1.3), D-penicillamine and its metabolites could not be observed in patient samples, even in pooled concentrated plasma. However, the application of the method described above should enable NMR to provide a wide variety of useful information on plasma, such as the metabolic pattern of endogenous components in normal or disease states where concentrations are greater than 0.1 mM.

5.3.3 IN VITRO METABOLISM

10,000 g liver fraction gave similar problems to those of plasma in ^{1}H NMR analysis. However, acid extracted samples used in the study indicate that ^{1}H NMR spectroscopy may be a useful method in *in vitro* metabolism studies. *In vitro* studies involved incubation of the parent drug with liver microsomes, soluble fractions, or both. The drug and/or its metabolites can be analysed by ^{1}H NMR quantitatively or qualitatively and may be a useful complement to kinetic studies *in vivo*. *In vitro* metabolism studies by ^{1}H NMR are well suited for exploring the metabolism of new drugs

providing the parent drug and its metabolites give suitable NMR signals and sensitivity problems can be overcome. An advantage is the possibility of examining endogenous and exogenous metabolites simultaneously.

^{1}H NMR spectroscopy can also indicate *in vivo* enzyme induction of PB or 3MC using a model substrate. Aminopyrine (DMAP) used as a model substrate was metabolised rapidly by PB induced enzymes, i.e. cytochrome P-450. Daunorubicin (DA) metabolism was found to be induced by both PB and 3MC which gave different metabolic products.

It is well known that many drugs have the capability of inducing one or more of the drug metabolising enzymes. Metabolism can lead to either more polar and less pharmacologically active (toxic) metabolites or to potentially toxic compounds such as epoxides and arene oxides. "Reactive" metabolites or intermediates are generally formed by the action of microsomal cytochromes, although there are some examples where the metabolic activation is dependent on the activity of conjugating enzymes. Pretreatment of animals with an inducer can increase the toxicity of drugs, at least in part, by enhancing the activity of the microsomal cytochromes which produce toxic metabolism. Thus inhibiting enzyme activity may diminish the toxicity. On the other hand, the toxicity of drugs which are metabolised to non-toxic products will be decreased by enzyme induction. The balance of toxifying and detoxifying pathways thus depends on the balance of various enzyme activities. Since ^{1}H NMR spectroscopy has proved to be a useful tool in studying *in vitro* metabolism and enzyme induction, it has potential in the study of toxifying and detoxifying pathways.

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